Guerts van Kessel, W. S. M., Hax, W. M. A., Demel, R. A., & deGier, J. (1977) *Biochim. Biophys. Acta 486*, 524–530.
Kates, M. (1972) *Techniques of Lipidology*, North-Holland, Amsterdam.
Kawanami, J. (1971) *Chem. Phys. Lipids 7*, 159–172.
Kawanami, J., Kimura, A., & Otsuka, H. (1968) *Biochim. Biophys. Acta 152*, 808–810.
Kenyon, C. N. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 281–313, Plenum Press, New York.
Kenyon, C. N., & Gray, A. M. (1974) *J. Bacteriol. 120*, 131–138.
Knaff, D. B., Olson, J. M., & Prince, R. C. (1979) *FEBS Lett. 98*, 285–289.
Knoche, H. W., & Shively, J. M. (1972) *J. Biol. Chem. 247*, 170–178.
Knudsen, E., Jantzen, E., Bryn, K., Ormerod, J. G., & Sirevog, R. (1982) *Arch. Microbiol. 135*, 149–154.
Nagai, M. (1968) *Arch. Biochem. Biophys. 126*, 426–435.
Olson, J. M. (1980) *Biochim. Biophys. Acta 594*, 33–51.
Olson, J. M. (1981) *Biochim. Biophys. Acta 637*, 185–188.
Olson, J. M., & Thornber, J. P. (1979) in *Membrane Proteins in Energy Transduction* (Capaldi, R. D., Ed.) pp 279–340, Marcel Dekker, New York.
Olson, J. M., Philipson, K. D., & Sauer, K. (1973) *Biochim. Biophys. Acta 292*, 206–217.
Poe, M. (1973) *J. Biol. Chem. 248*, 7025–7032.
Prome, J.-C., Lacave, C., & Laneele, M.-A. (1969) *C. R. Hebd. Seances Acad. Sci., Ser. C 269*, 1664–1667.
Savitsky, A., & Golay, M. J. E. (1964) *Anal. Chem. 36*, 1627–1639.
Schmidt, K. (1980) *Arch. Microbiol. 124*, 21–31.
Shaw, N. (1968) *Biochim. Biophys. Acta 152*, 427–428.
Staehlin, L. A., Golecki, J. R., & Drews, G. (1980) *Biochim. Biophys. Acta 589*, 30–45.
Swarthoff, T., Kramer, H. J. M., & Amesz, J. (1982) *Biochim. Biophys. Acta 681*, 354–358.
Vaskovsky, V. E., & Kostetsky, E. Y. (1968) *J. Lipid Res. 9*, 396.
Wilkinson, S. G. (1972) *Biochim. Biophys. Acta 270*, 1–17.

Metabolism of 4-Pentenoic Acid and Inhibition of Thiolase by Metabolites of 4-Pentenoic Acid†

Horst Schulz

ABSTRACT: The metabolism of 4-pentenoic acid, a hypoglycemic agent and inhibitor of fatty acid oxidation, has been studied in rat heart mitochondria. Confirmed was the conversion of 4-pentenoic acid to 2,4-pentadienoyl coenzyme A (CoA), which either is directly degraded via β-oxidation or is first reduced in a NADPH-dependent reaction before it is further degraded by β-oxidation. At pH 6.9, the NADPH-dependent reduction of 2,4-pentadienoyl-CoA proceeds 10 times faster than its degradation by β-oxidation. At pH 7.8, this ratio is only 2 to 1. The direct β-oxidation of 2,4-pentadienoyl-CoA leads to the formation of 3-keto-4-pentenoyl-CoA, which is highly reactive and spontaneously converts to another 3-ketoacyl-CoA derivative (compound X). 3-Keto-4-pentenoyl-CoA is a poor substrate of 3-ketoacyl-CoA thiolase (EC 2.3.1.16) whereas compound X is not measurably acted upon by this enzyme. The effects of several metabolites of 4-pentenoic acid on the activity of 3-ketoacyl-CoA thiolase were studied. 2,4-Pentadienoyl-CoA is a weak inhibitor of this enzyme that is protected against the inhibition by acetoacetyl-CoA. The most effective inhibitor of 3-ketoacyl-CoA thiolase was found to be 3-keto-4-pentenoyl-CoA, which inhibits the enzyme in both a reversible and irreversible manner. The reversible inhibition is possibly a consequence of the inhibitor being a poor substrate of 3-ketoacyl-CoA thiolase. It is concluded that 4-pentenoic acid is metabolized in mitochondria by two pathways. The minor yields 3-keto-4-pentenoyl-CoA, which acts both as a reversible and as a irreversible inhibitor of 3-ketoacyl-CoA thiolase and consequently of fatty acid oxidation.

4-Pentenoic acid is a hypoglycemic agent and an inhibitor of fatty aicd oxidation [for a recent review, see Billington et al. (1978a,b)]. Although the inhibition of fatty acid oxidation by 4-pentenoic acid has been well documented (Senior et al., 1968; Brendel et al., 1969; Williamson et al., 1970), the molecular basis of this inhibition has not been fully elucidated. Bressler et al. (1969) have concluded from their studies that metabolites of 4-pentenoic acid, which are slowly or not at all metabolized, sequester coenzyme A (CoA) and carnitine and thereby inhibit fatty acid oxidation. In contrast, Sherratt and co-workers (Billington et al., 1978a) have proposed that one or several metabolites of 4-pentenoic acid inhibit at least one of the enzymes of β-oxidation in a reversible fashion. Although these authors have observed the inhibition of isolated acetoacetyl-CoA thiolase (EC 2.3.1.9) by 2,4-pentadienoyl-CoA (Holland et al., 1973), they have been unable to detect the inactivation of the same enzyme in mitochondria preincubated with 4-pentenoic acid (Billington et al., 1978a). However, Fong & Schulz (1978) have demonstrated the inactivation of both 3-ketoacyl-CoA thiolase (EC 2.3.1.16) and acetoacetyl-CoA thiolase when coupled rat heart mitochondria were incubated with 4-pentenoic acid. Preliminary evidence pointed to 3-keto-4-pentenoyl-CoA as the inhibitory metabolite of 4-pentenoic acid (Schulz & Fong, 1981). However, a definite

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clarification of the mechanism by which 4-pentenoic acid inhibits thiolase and thus fatty acid oxidation is still lacking. This situation is partly due to the uncertainty about the metabolism of 4-pentenoic acid. It is well established that 4-pentenoic acid can be converted to its CoA derivative and then dehydrogenated to 2,4-pentadienoyl-CoA (Holland et al., 1973; Corredor, 1975). The observed formation of acryloylcarnitine from 4-pentenoylcarnitine (Brendel et al., 1969) and the observed action of crotonase (EC 4.2.1.17) and L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) on 2,4-pentadienoyl-CoA (Holland et al., 1973) suggest that 2,4-pentadienoyl-CoA may be metabolized via β-oxidation. However, the further metabolism of acryloylcarnitine or acryloyl-CoA has not been elucidated. More recently, Hiltunen (1978) and Hiltunen et al. (1980) have observed that 4-pentenoic acid is metabolized via propionyl-CoA to intermediates of the tricarboxylic acid cycle. The key reaction in this pathway is the NADPH-dependent reduction of 2,4-pentadienoyl-CoA to 3-pentenoyl-CoA or 2-pentenoyl-CoA (Borreback et al., 1980; Hiltunen & Davis, 1981). It thus appears that 2,4-pentadienoyl-CoA can be metabolized by two different pathways. In this paper, I present more detailed information on the metabolism of 4-pentenoic acid and on the mechanism by which 3-keto-4-pentenoyl-CoA, a metabolite of 4-pentenoic acid, inhibits 3-ketoacyl-CoA thiolase.

Experimental Procedures

Materials. CoASH, NAD, and NADPH were purchased from P-L Biochemicals, Inc. 4-Pentenoic acid was obtained from Fluka A. G., Switzerland. L-3-Hydroxyacyl-CoA dehydrogenase, lactate dehydrogenase, sodium pyruvate, and other standard biochemicals were bought from Sigma Chemical Co. Beef liver crotonase was purified as described by Steinman & Hill (1975). 3-Ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase were isolated and purified from pig heart by the procedure of Staack et al. (1978). Rat heart mitochondria were isolated as described by Chappell & Hansford (1969). For the preparation of a soluble mitochondrial extract, rat heart mitochondria were diluted with 0.5 M potassium phosphate (pH 7.6) to a final concentration of 10 mg of mitochondrial protein/mL of 20 mM potassium phosphate (pH 7.6). This suspension of mitochondria was sonicated at 4 °C with a Branson sonifier equipped with a microtip 6 times for 5 s each. The resulting suspension was centrifuged at 4 °C for 60 min at 100000*g*, and the supernatant was used as a source of β-oxidation enzymes. 2,4-Pentadienoic acid was synthesized from malonic acid and acrolein as described by Kohler & Butler (1926). The CoA derivatives of 2,4-pentadienoic acid and 4-pentenoic acid were prepared in 70–80% yield by the mixed-anhydride method of Goldman & Vagelos (1961). The molar extinction coefficient of 2,4-pentadienoyl-CoA was determined to be 13.5×10^3 M^{-1} cm^{-1} at $\lambda = 285$ nm. Acetoacetyl-CoA was synthesized as described by Seubert (1960). The concentrations of CoA derivatives were determined by the method of Ellman (1959) after cleaving the thio ester bond with hydroxylamine at pH 7.

Enzymatic Conversion of 2,4-Pentadienoyl-CoA to 3-Keto-4-pentenoyl-CoA. A standard reaction mixture for the conversion of 2,4-pentadienoyl-CoA to 3-keto-4-pentenoyl-CoA contained 0.1 M Tris-HCl[1] (pH 8.2), 25 mM $MgCl_2$, 35 μM 2,4-pentadienoyl-CoA, 0.14 mM NAD, 10 mM sodium pyruvate, lactate dehydrogenase (1 unit), L-3-hydroxyacyl-CoA dehydrogenase (0.7 unit), and crotonase (5 units). The reaction was started by the addition of crotonase. The absorbance at 334 nm reached a maximum of 0.18 within 7 min and thereafter remained constant for 10 min. In order to obtain an approximate value for the extinction coefficient of the Mg^{2+}–enolate complex of 3-keto-4-pentenoyl-CoA, this compound was generated as described above except that pyruvate and lactate dehydrogenase were deleted from the reaction mixture. Upon addition of EDTA the absorbance at 334 nm decreased due to the disappearance of the Mg^{2+}–enolate complex of 3-keto-4-pentenoyl-CoA. Addition of pyruvate and lactate dehydrogenase resulted in the oxidation of NADH and a corresponding absorbance decrease at 340 nm. On the basis of the observed absorbance changes and on $\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1} for NADH, an approximate value of 20×10^3 M^{-1} cm^{-1} was calculated for the extinction coefficient of the Mg^{2+}–enolate complex of 3-keto-4-pentenoyl-CoA. A value of 17×10^3 M^{-1} cm^{-1} was obtained on the basis of the measured decrease in absorbance at 285 nm ($\epsilon = 13.5 \times 10^3$ M^{-1} cm^{-1}) and the corresponding increase in absorbance at 334 nm during the conversion of 2,4-pentadienoyl-CoA to 3-keto-4-pentenoyl-CoA.

Enzyme and Protein Assays. 3-Ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase were assayed by following spectrophotometrically the decrease in absorbance at 303 nm due to the disappearance of the Mg^{2+}–enolate complex of acetoacetyl-CoA as described in principle by Lynen & Ochoa (1953). A standard assay mixture contained 0.1 M Tris-HCl (pH 8.2), 25 mM $MgCl_2$, 33 μM acetoacetyl-CoA, 90 μM CoASH, and 3-ketoacyl-CoA thiolase (3 milliunits). The NADPH-dependent reduction of 2,4-pentadienoyl-CoA was measured spectrophotometrically at 340 nm. A typical assay mixture contained 0.13 M Tris-HCl (pH 8.2), 40 μM 2,4-pentadienoyl-CoA, 90 μM NADPH, and a soluble extract of rat heart mitochondria (0.13 mg of protein/mL). For identification of the product of the reduction of 2,4-pentadienoyl-CoA, the reaction was terminated by acidification to pH 1. After 50 min, the pH was adjusted to 8.5, and the following additions were made: 10 mM sodium pyruvate, 0.13 mM NAD, 25 mM $MgCl_2$, and lactate dehydrogenase (1 unit). The reaction was started by the addition of L-3-hydroxyacyl-CoA dehydrogenase (0.7 unit) to the sample in the measuring beam only. A unit of enzyme activity is defined as the amount that catalyzes the conversion of 1 μmol of substrate to product per minute. Protein concentrations were determined according to Lowry et al. (1951).

Inibition of 3-Ketoacyl-CoA Thiolase by 2,4-Pentadienoyl-CoA or 3-Keto-4-pentenoyl-CoA. 3-Ketoacryl-CoA thiolase (3 μg) was incubated for 30 s with 1 mL of a solution containing either 9 μM 3-keto-4-pentenoyl-CoA or 35 μM 2,4-pentadienoyl-CoA. The incubation mixtures were rapidly filtered through Sephadex G-50 (10 mL) as described in principle by Penefsky (1977). Aliquots (0.3 mL) of the filtered, as well as unfiltered, solutions were added to 0.3 mL of thiolase assay mixtures that contained the components listed above, and thiolase activities were determined.

Results

Metabolism of 4-Pentenoic Acid. The reported conversions of 4-pentenoic acid to 4-pentenoyl-CoA and of 4-pentenoyl-CoA to 2,4-pentadienoyl-CoA (Holland et al., 1973; Corredor, 1975) were confirmed with rat heart mitochondria as a source of enzymes (data not shown). As reported by Holland et al. (1973), crotonase did not hydrate 2,4-pentadienoyl-CoA to a measurable degree. However, 2,4-pentadienoyl-CoA was metabolized by crotonase plus L-3-hydroxyacyl-CoA de-

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

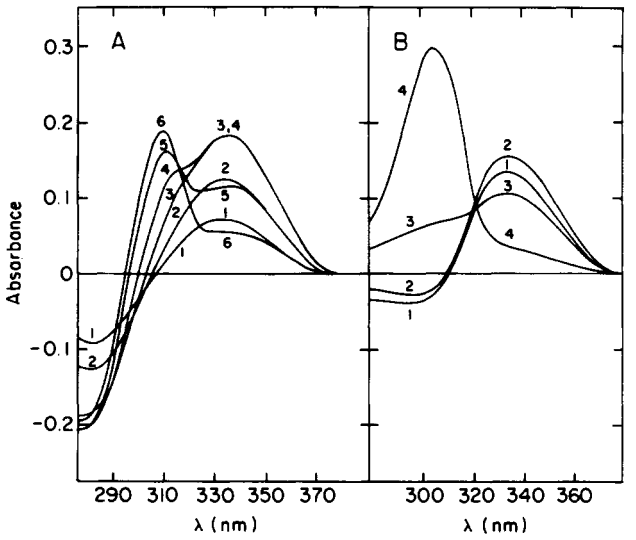

FIGURE 1: β-Oxidation of 2,4-pentadienoyl-CoA. (A) Spectral changes associated with the conversion of 2,4-pentadienoyl-CoA to 3-keto-4-pentenoyl-CoA and compound X catalyzed by crotonase and L-3-hydroxyacyl-CoA dehydrogenase after (1) 1 min 15 s, (2) 2 min 45 s, (3) 7 min 40 s, (4) 15 min, (5) 30 min, and (6) 47 min. For experimental details, see Experimental Procedures. The reference cuvette contained all components of the incubation mixture except for crotonase. (B) Spectral changes associated with the cleavage of 3-keto-4-pentenoyl-CoA catalyzed by 3-ketoacyl-CoA thiolase. Difference spectrum of 3-keto-4-pentenoyl-CoA [represented by curve 3 in (A)] plus 55 μM CoASH vs. 3-keto-4-pentenoyl-CoA plus 55 μM CoASH and 3-ketoacyl-CoA thiolase (0.3 unit) after (1) 1 min, (2) 2 min, (3) 8 min 30 s, and (4) 24 min.

hydrogenase in the presence of NAD and $MgCl_2$ to a compound with an absorbance maximum at 334 nm (see Figure 1A). This reaction was accelerated when pyruvate and lactate dehydrogenase were added to reoxidize NADH formed during the reaction (data not shown). The compound with an absorbance maximum at 334 nm was presumably the Mg^{2+}–enolate complex of 3-keto-4-pentenoyl-CoA. This assignment is based on the following: (a) the disappearance of the chromophore of 2,4-pentadienoyl-CoA at 285 nm and the parallel appearance of the expected chromophore at 334 nm of the Mg^{2+}–enolate complex of 3-keto-4-pentenoyl-CoA (see Figure 1A); (b) the complete disappearance of the chromophore at 334 nm when EDTA in excess of $MgCl_2$ was added to the incubation mixture (data not shown); and (c) the disappearance of the chromophore at 334 nm in the presence of 3-ketoacyl-CoA thiolase and CoASH shown in Figure 1B and discussed below. 3-Keto-4-pentenoyl-CoA was unstable under the conditions at which it was formed as evidenced by the disappearance of its chromophore at 334 nm and the appearance of a new absorbance close to 310 nm. The conversion of 3-keto-4-pentenoyl-CoA to the compound with an absorbance at 310 nm (compound X) was faster in the absence of $MgCl_2$ and at lower pH (data not shown). These observations suggest that the keto form and not the enolate form of 3-keto-4-pentenoyl-CoA is a reactant in the formation of compound X. Separation by ultrafiltration of the enzymes from 3-keto-4-pentenoyl-CoA did not prevent its conversion to compound X (data not shown). Thus, the formation of compound X from 3-keto-4-pentenoyl-CoA is not enzyme catalyzed. Since addition of EDTA in excess of $MgCl_2$ to compound X resulted in the disappearance of the absorbance at 310 nm, compound X is most likely the Mg^{2+}–enolate complex of a 3-ketoacyl-CoA compound. The rapid formation of a compound with an absorbance maximum close to 310 nm was observed when 3-keto-4-pentenoyl-CoA was reacted with 55 μM CoASH at pH 8.2 (data not shown). This observation as well as the spontaneous formation of compound X attest to the instability of 3-keto-4-pentenoyl-CoA. Because of the rapid noncatalyzed reaction of 3-keto-4-pentenoyl-CoA with CoASH, the thiolytic cleavage of this compound was determined by measuring the difference spectrum of 3-keto-4-pentenoyl-CoA plus CoASH vs. 3-keto-4-pentenoyl-CoA plus CoASH and 3-ketoacyl-CoA thiolase. As shown in Figure 1B, 3-keto-4-pentenoyl-CoA present in the reference cuvette was cleaved by 3-ketoacyl-CoA thiolase as evidenced by the appearance of an absorption band at 334 nm (see spectra 1 and 2 in Figure 1B). This absorption band disappeared with time because 3-keto-4-pentenoyl-CoA in the measuring cuvette reacted with CoASH to yield a compound with an absorption band at 303 nm. Clearly, 3-keto-4-pentenoyl-CoA is a substrate for 3-ketoacyl-CoA thiolase, although a poor one, since its rate of thiolytic cleavage was less than 3% of the rate observed with acetoacetyl-CoA. The rate at which compound X was cleaved was less than 0.1% of that seen with acetoacetyl-CoA.

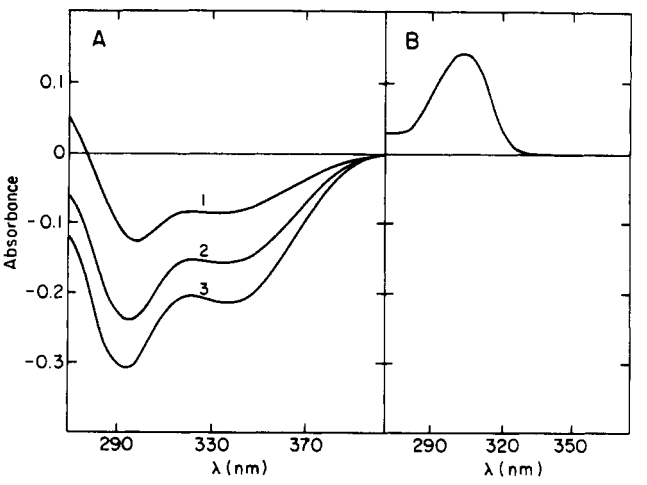

FIGURE 2: NADPH-dependent reduction of 2,4-pentadienoyl-CoA catalyzed by a soluble extract of rat heart mitochondria. (A) Difference spectrum of a complete incubation mixture (for details see Experimental Procedures) vs. the same mixture without enzyme after (1) 8 min, (2) 20 min, and (3) 58 min. Spectral changes observed when the product formed in the reaction mixture represented by curve 3 of (A) was dehydrogenated by NAD in the presence of L-3-hydroxyacyl-CoA dehydrogenase. For experimental details see Experimental Procedures.

A reaction between 2,4-pentadienoyl-CoA and NADPH was catalyzed by a soluble extract of rat heart mitochondria (see Figure 2A). The parallel decreases of the absorbances below 300 and at 340 nm are indicative of the reduction of 2,4-pentadienoyl-CoA by NADPH. The observed stoichiometry of the reduction of 2,4-pentadienoyl-CoA ($\epsilon = 1.35 \times 10^4$ M^{-1} cm^{-1}) and of the oxidation of NADPH ($\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}) agrees with the reduction of one of the two diene double bonds. It is likely that the reduction of 2,4-pentadienoyl-CoA is catalyzed by the NADPH-dependent 2,4-dienoyl-CoA reductase first described by Kunau & Dommes (1978). If so, the product of the reaction would be 3-pentenoyl-CoA, which would be converted to 3-hydroxypentanoyl-CoA due to the presence of *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase and crotonase in the mitochondrial extract. So that this sequence of enzyme-catalyzed reactions could be proved, the reduction of 2,4-pentadienoyl-CoA by NADPH was terminated after 1 h by acidification. The formation of 3-hydroxypentanoyl-CoA was demonstrated by its conversion to the Mg^{2-}enoylate complex of 3-ketopentanoyl-CoA, which has an absorbance maximum at 303 nm (see Figure 1B) that disappeared when

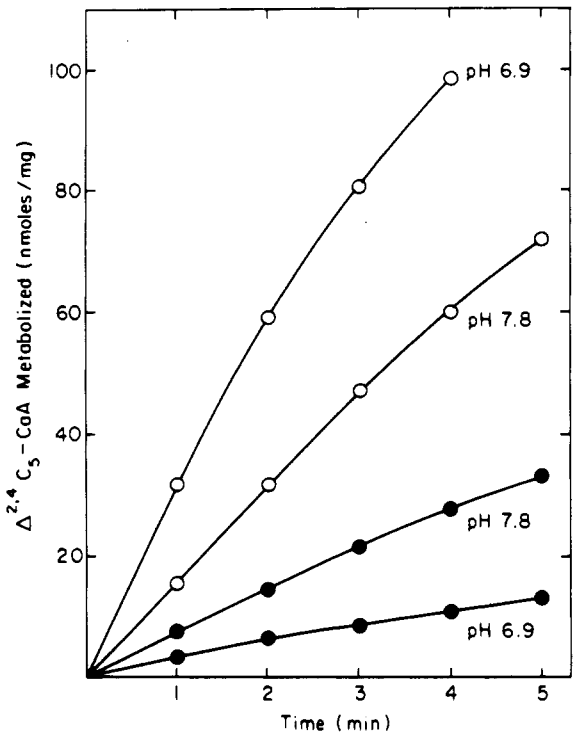

FIGURE 3: Metabolism of 2,4-pentadienoyl-CoA by rat heart mitochondria. All incubation mixtures contained 0.2 M potassium phosphate, 40 μM 2,4-pentadienoyl-CoA, 0.15% Triton X-100, and rat heart mitochondria (0.22 mg of protein/mL). (○) NADPH-dependent reduction of 2,4-pentadienoyl-CoA. Assay mixtures contained additionally 90 μM NADPH. (●) β-Oxidation of 2,4-pentadienoyl-CoA. Assay mixtures contained additionally 10 mM sodium pyruvate, 0.12 mM NAD, 90 μM CoASH, and lactate dehydrogenase (65 units/mL).

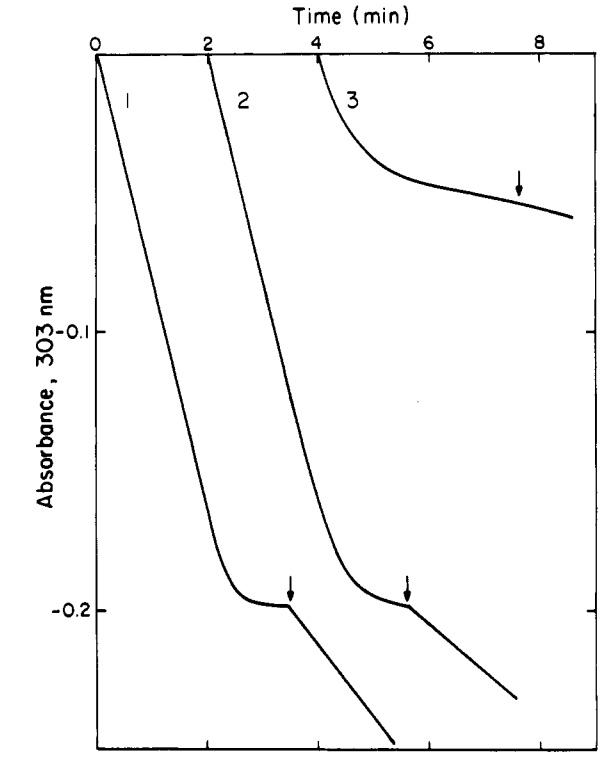

FIGURE 4: Effect of 2,4-pentadienoyl-CoA and its metabolites on activity of 3-ketoacyl-CoA thiolase. The incubation mixtures contained the following: (1) all the components of the thiolase assay (for details see Experimental Procedures) plus 0.23 mM NAD, L-3-hydroxyacyl-CoA dehydrogenase (0.6 unit), and crotonase (30 milliunits); (2) 7 μM 2,4-pentadienoyl-CoA plus all components listed under (1) except for NAD; (3) all components listed under (1) plus 7 μM 2,4-pentadienoyl-CoA. At the times indicated by the arrows additional acetoacetyl-CoA was added.

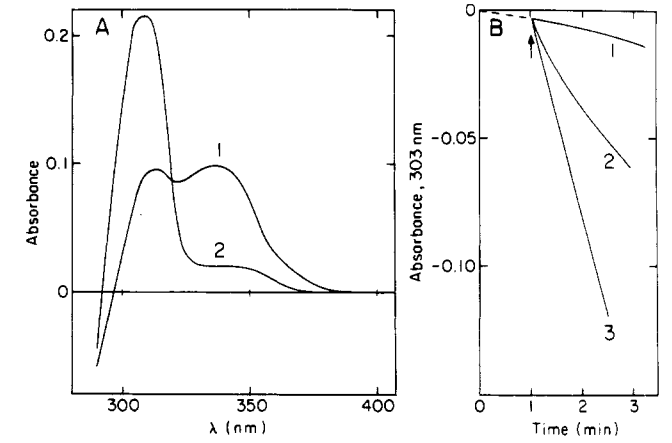

FIGURE 5: Effect of 3-keto-4-pentenoyl-CoA and compound X on activity of 3-ketoacyl-CoA thiolase. (A) (1) Spectrum of 3-keto-4-pentenoyl-CoA containing some compound X; (2) spectrum of compound X containing little 3-keto-4-pentenoyl-CoA. Both compounds were formed enzymatically as described in detail under Experimental Procedures. (B) Thiolytic activity of 3-ketoacyl-CoA thiolase in the presence of (1) approximately 2 μM 3-keto-4-pentenoyl-CoA [represented by spectrum 1 in (A)], (2) approximately 6 μM compound X [represented by spectrum 2 in (A)], or (3) 17 μM 2,4-pentadienoyl-CoA. The assays were started by the simultaneous addition of CoASH and enzyme. Details of the assay are given under Experimental Procedures.

EDTA in excess of $MgCl_2$ was added to the incubation mixture (data not shown). The rate at which 2,4-pentadienoyl-CoA was reduced by NADPH was compared with the rate at which it was metabolized by β-oxidation. As shown in Figure 3, the reduction of 2,4-pentadienoyl-CoA at pH 6.9 was 10 times faster than its rate of β-oxidation. However, at pH 7.8, the reduction was only twice as fast as its β-oxidation. This observation suggests that the direct β-oxidation, which leads to the formation of 3-keto-4-pentenoyl-CoA, is a significant metabolic pathway of 4-pentenoic acid in rat heart mitochondria.

Inhibition of 3-Ketoacyl-CoA Thiolase by Metabolites of 4-Pentenoic Acid. 4-Pentenoyl-CoA, the first metabolite of 4-pentenoic acid, did not affect the activity of 3-ketoacyl-CoA thiolase. Even 2,4-pentadienoyl-CoA, when added to a complete thiolase assay mixture, did not inhibit the reaction (see Figure 4). However, when 2,4-pentadienoyl-CoA plus crotonase, L-3-hydroxyacyl-CoA dehydrogenase, and NAD were present in the thiolase assay mixture, 3-ketoacyl-CoA thiolase was rapidly inactivated (see Figure 4). A similar result was obtained with acetoacetyl-CoA thiolase from pig heart. This observation suggests that a metabolite of 2,4-pentadienoyl-CoA, possibly 3-keto-4-pentenoyl-CoA, is a strong inhibitor of 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase. Since Holland et al. (1973) had observed an inhibition of 3-ketoacyl-CoA thiolase by 2,4-pentadienoyl-CoA, the effect of this compound on 3-ketoacyl-CoA thiolase was studied as a function of the preincubation time. 2,4-Pentadienoyl-CoA (24 μM) caused the inactivation of 78% of 3-ketoacyl-CoA thiolase within 10 min. However, in the presence of acetoacetyl-CoA (28 μM) the activity of thiolase decreased by only 16%. This protection explains why 2,4-pentadienoyl-CoA did not inactivate thiolase when added to a complete assay mixture that contained acetoacetyl-CoA. The effect of preformed 3-keto-4-pentenoyl-CoA on 3-ketoacyl-CoA thiolase was studied. When a mixture of 3-keto-4-pentenoyl-CoA (4.5 μM) and compound X (see Figure 5A, spectrum 1) was present in

Table I: Inhibition of 3-Ketoacyl-CoA Thiolase by Metabolites of 4-Pentenoic Acid[a]

additions	filtered[b]	thiolase act. (units/mg)	rel act. (%)
none	no	6.6	100
2,4-pentadienoyl-CoA (35 μM)	no	5.6	85
3-keto-4-pentenoyl-CoA (9 μM)	no	0 (0.3)[c]	0 (4)[c]
none	yes	4.9	100
2,4-pentadienoyl-CoA (35 μM)	yes	3.7	76
3-keto-4-pentenoyl-CoA (9 μM)	yes	0.7	14

[a] For experimental details see Experimental Procedures. [b] Filtered through a Sephadex G-50 column as described under Experimental Procedures. [c] Thiolase activity 3 min after starting the assay.

a thiolase assay mixture, 3-ketoacyl-CoA thiolase was fully inhibited (see Figure 5B, curve 1). However, when 3-keto-4-pentenoyl-CoA was almost completely converted to compound X (see Figure 5A, spectrum 2), only a slight inhibition of 3-ketoacyl-CoA thiolase was observed (see Figure 5B, curve 2). Clearly, the inhibition of 3-ketoacyl-CoA thiolase was caused by 3-keto-4-pentenoyl-CoA and not by compound X. The inhibition of 3-ketoacyl-CoA thiolase was complete within 10 s, the time necessary to start the thiolase assay. However, 2 min after the start of the reaction, a slight reactivation of the enzyme appeared to take place (see Figure 5B, curve 1). The thiolase assay was followed for longer periods of time and at two different concentrations of 3-keto-4-pentenoyl-CoA, in order to study this phenomenon. 3-Ketoacyl-CoA thiolase (1.5 μg/mL) was completely inactive after the addition of 4.5 μM inhibitor, but after 3 min, it exhibited 4% of its original activity. Even in the presence of 1.5 μM inhibitor, the enzyme was initially inactive, but it regained 25% of its activity within 4 min. It thus appears that at least part of the inhibition of 3-ketoacyl-CoA thiolase caused by 3-keto-4-pentenoyl-CoA is reversible. So that this tentative conclusion could be proved, the inhibition of this enzyme caused within 30 s by 3-keto-4-pentenoyl-CoA or 2,4-pentadienoyl-CoA was determined before and after filtering the enzyme through Sephadex G-50. The time required to complete the Sephadex filtration was 5 min, and this delay and operational losses were the cause for the partial loss of enzyme activity. The data presented in Table I demonstrate that removal of 3-keto-4-pentenoyl-CoA from the enzyme solution after 30 s of preincubation resulted in the recovery of 14% of the original thiolase activity. However, 86% of the thiolase activity was irreversibly lost, most likely due to the covalent modification of the enzyme. The results shown in Table I emphasize that 3-ketoacyl-CoA thiolase is much more severely inhibited by 3-keto-4-pentenoyl-CoA than by 2,4-pentadienoyl-CoA.

Discussion

The results of this and previous studies (Bressler et al., 1969; Holland et al., 1973; Hiltunen & Davis, 1981) suggest that 4-pentenoic aicd can be metabolized intramitochondrially by two pathways as outlined in Figure 6. Common to both pathways are the activation of 4-pentenoic aicd to 4-pentenoyl-CoA, presumably catalyzed by medium-chain acyl-CoA synthetase, and the dehydrogenation of 4-pentenoyl-CoA to 2,4-pentadienoyl-CoA catalyzed by butyryl-CoA dehydrogenase. 2,4-Pentadienoyl-CoA can be directly degraded by β-oxidation as outlined in Figure 6A. Its hydration by crotonase to 3-hydroxy-4-pentenoyl-CoA is thermodynamically

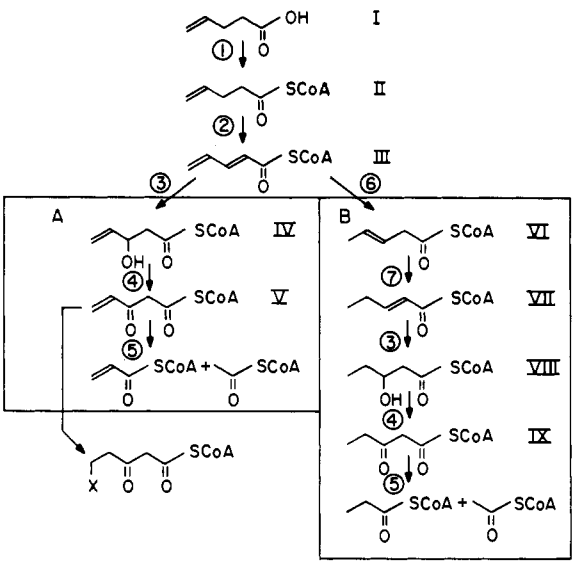

FIGURE 6: Degradative pathways of 4-pentenoic acid in mitochondria. (A) Minor pathway that yields acryloyl-CoA and acetyl-CoA. (B) Major pathway that yields propionyl-CoA and acetyl-CoA. The intermediates are as follows: (I) 4-pentenoic acid; (II) 4-pentenoyl-CoA; (III) 2,4-pentadienoyl-CoA; (IV) 3-hydroxy-4-pentenoyl-CoA; (V) 3-keto-4-pentenoyl-CoA; (VI) 3-pentenoyl-CoA; (VII) 2-pentenoyl-CoA; (VIII) 3-hydroxypentanoyl-CoA; (IX) 3-ketopentanoyl-CoA. Enzymes involved in the degradation of 4-pentenoic acid are as follows: (1) acyl-CoA synthetase; (2) acyl-CoA dehydrogenase; (3) crotonase; (4) L-3-hydroxyacyl-CoA dehydrogenase; (5) 3-ketoacyl-CoA thiolase; (6) 2,4-pentadienoyl-CoA reductase; (7) *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase.

unfavorable and does not proceed to a degree sufficient for direct measurements (Holland et al., 1973). However, when the hydration of 2,4-pentadienoyl-CoA is coupled to its NAD-dependent dehydrogenation catalyzed by L-3-hydroxylacyl-CoA dehydrogenase, the reaction can be easily followed spectrophotometrically. The product of this two-step reaction sequence is 3-keto-4-pentenoyl-CoA, which is a substrate, although a poor one, of 3-ketoacyl-CoA thiolase and thus is expected to be cleaved to acryloyl-CoA and acetyl-CoA. The formation of acryloylcarnitine from 4-pentenoic acid by a pigeon liver homogenate has been reported (Brendel et al., 1969). However, the further metabolism of acryloyl-CoA has not been elucidated. 3-Keto-4-pentenoyl-CoA reacts rapidly and nonenzymatically with CoASH to yield a 3-ketoacyl-CoA compound that, on the basis of its spectral properties, is devoid of the terminal double bond. It seems likely that CoASH had reacted at pH 8.2 with 3-keto-4-pentenoyl-CoA to yield a Michael adduct in which CoA is attached to carbon 5 of 3-ketopentanoyl-CoA by a thioether linkage. The slower conversion of 3-keto-4-pentenoyl-CoA to compound X possibly relfects the hydration of the double bond between carbons 4 and 5. If so, compound X might be 3-keto-5-hydroxypentanoyl-CoA, which apparently is not acted upon by 3-ketoacyl-CoA thiolase and whose further metabolism remains unresolved. The second pathway by which 2,4-pentadienoyl-CoA is metabolized is summarized in Figure 6B. The crucial step is the NADPH-dependent reduction of 2,4-pentadienoyl-CoA. The product of this reaction is 3-pentanoyl-CoA if the reaction is catalyzed by 2,4-dienoyl-CoA reductase as originally described by Kunau & Dommes (1978). 3-Pentenoyl-CoA can be converted by *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase to 2-pentenoyl-CoA, which can be degraded via β-oxidation to propionyl-CoA and acetyl-CoA. In vitro measurements aimed at assessing the entry of 2,4-pentadienoyl-CoA into the two pathways indicated that at pH 6.9 only 10% of this compound was degraded by direct β-oxidation.

However, at pH 7.8, this percentage was 33%. Since these values were obtained by in vitro measurements, they do not take into account the possible preferential channeling of 2,4-pentadienoyl-CoA into one of these pathways. However, it appears that pathway A (see Figure 6) is the minor pathway that yields the metabolite responsible for the inhibition of fatty acid oxidation. This conclusion is supported by the observation that the inhibition of fatty acid oxidation by 4-pentenoic acid in rat hepatocytes is relieved by feeding of clofibrate, which causes a several-fold increase in the activity of the NADPH-dependent 2,4-dienoyl-CoA reductase (Borreback et al., 1980).

Since the inhibition of fatty acid oxidation by 4-pentenoic acid in rat heart mitochondria was found to be a consequence of the inactivation of 3-ketoacyl-CoA thiolase (Fong & Schulz, 1978), the effect of metabolites of 4-pentenoic acid on 3-ketoacyl-CoA thiolase was studied. This study established that 3-keto-4-pentenoyl-CoA, a metabolite of 4-pentenoic acid, is an effective inhibitor of 3-ketoacyl-CoA thiolase. 2,4-Pentadienoyl-CoA in comparison is a poor inhibitor of 3-ketoacyl-CoA thiolase. 3-Keto-4-pentenoyl-CoA inhibits 3-ketoacyl-CoA thiolase both reversibly and irreversibly. The reversible inhibition is possibly due to 3-keto-4-pentenoyl-CoA binding to the active site of the enzyme, but being only slowly converted to product. If this conclusion is correct, 3-keto-4-pentenoyl-CoA must bind to the enzyme very tightly because it inhibits the enzyme completely at concentrations of inhibitor and substrate of 4.5 and 24 μM, respectively. The irreversible inhibition of 3-ketoacyl-CoA thiolase could be the consequence of several different reactions between enzyme and inhibitor. The most likely reactions are (a) the covalent attachment of the complete inhibitor molecule to the enzyme or (b) the formation of a covalent bond between the enzyme and the acryloyl residue derived from 3-keto-4-pentenoyl-CoA. The latter reaction might take place while the acryloyl residue is temporarily attached to the essential sulfhydryl group of the enzyme. Further experiments are needed to distinguish between these two and other possibilities for the irreversible inhibition of 3-ketoacryl-CoA thiolase by 3-keto-4-pentenoyl-CoA. Unfortunately, the instability of 3-keto-4-pentenoyl-CoA makes such a study a difficult one.

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References

Billington, D., Osmundson, H., & Sherratt, H. S. A. (1978a) *Biochem. Pharmacol. 27*, 2879–2890.

Billington, D., Osmundson, H., & Sherratt, H. S. A. (1978b) *Biochem. Pharmacol. 27*, 2891–2900.

Borreback, B., Osmundson, H., & Bremer, J. (1980) *Biochem. Biophys. Res. Commun. 93*, 1173–1180.

Brendel, K., Corredor, C. F., & Bressler, R. (1969) *Biochem. Biophys. Res. Commun. 34*, 340–347.

Bressler, R., Corredor, C. F., & Brendel, K. (1969) *Pharmacol. Rev. 21*, 105–130.

Chappell, J. B., & Hansford, R. G. (1969) in *Subcellular Components* (Birnie, G. D., Ed.) 2nd ed., pp 77–91, Butterworths, London.

Corredor, C. P. (1975) in *Hypoglycin* (Kean, E. A., Ed.) pp 93–107, Academic Press, New York.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Fong, J. C., & Schulz, H. (1978) *J. Biol. Chem. 253*, 6917–6922.

Goldman, P., & Vagelos, P. R. (1961) *J. Biol. Chem. 236*, 2620–2623.

Hiltunen, J. K. (1978) *Biochem. J. 170*, 241–247.

Hiltunen, J. K., & Davis, E. J. (1981) *Biochem. J. 194*, 427–432.

Hiltunen, J. K., Kauppinen, R. A., Nuntinen, E. M., Peuhkurinen, K. J., & Hassinen, I. E. (1980) *Biochem. J. 188*, 725–729.

Holland, P. C., Senior, A. E., & Sherratt, H. S. A. (1973) *Biochem. J. 136*, 173–184.

Kohler, E. P., & Butler, F. R. (1926) *J. Am. Chem. Soc. 48*, 1036–1048.

Kunau, W. H., & Dommes, P. (1978) *Eur. J. Biochem. 91*, 533–544.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lynen, F., & Ochoa, S. (1953) *Biochim. Biophys. Acta 12*, 299–314.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Schulz, H., & Fong, J. C. (1981) *Methods Enzymol. 72*, 604–610.

Senior, A. E., Robson, B., & Sherratt, H. S. A. (1968) *Biochem. J. 110*, 511–519.

Seubert, W. (1960) in *Biochemical Preparations* (Lardy, A. H., Ed.) Vol. 7, p 80, Wiley, New York.

Staack, H., Binstock, J. F., & Schulz, H. (1978) *J. Biol. Chem. 253*, 1827–1831.

Steinman, H., & Hill, R. L. (1975) *Methods Enzymol. 35*, 136–151.

Williamson, J. R., Rostand, S. G., & Peterson, M. J. (1970) *J. Biol. Chem. 245*, 3242–3251.