STUDIES ON THE AEROBIC OXIDATION OF FATTY ACIDS BY BACTERIA

V. Caprate Oxidation by Cell-Free Extracts of *Pseudomonas fluorescens*[1]

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The pathway of caprate oxidation has been studied previously with resting cell suspensions of *Serratia marcescens* using the technique of simultaneous adaptation (Silliker and Rittenberg, 1951) and metabolic inhibitors (Silliker and Rittenberg, 1952; Waltman and Rittenberg, 1954). The data obtained, while showing the inadequacy of certain classically held concepts, failed to reveal the exact nature of the intermediates involved. Attempts to obtain cell-free extracts of *S. marcescens* capable of caprate oxidation for further investigation of this problem were largely unsuccessful. It was found, however, that active cell-free preparations could be obtained from *Pseudomonas fluorescens*, and this paper deals with the results obtained using such extracts.

METHODS

The organism employed was a strain (P-6) of *P. fluorescens*, a soil isolate. It was cultured in a liquid mineral salts medium with 0.4 per cent disodium malonate as the carbon source. Cells were harvested with a Sharples centrifuge, washed with distilled water, and dried *in vacuo* over anhydrous calcium sulfate. Cell-free extracts were obtained by hand grinding the dry cells with 1 to 1½ parts of 0.05 M phosphate buffer, pH 6.1, and 3 parts of pyrex glass powder in a chilled mortar. The resulting paste was suspended in and extracted with 20 parts of cold buffer and centrifuged in the cold for 30 minutes at 20,000 × G. The clear supernatant was used as such (crude extract) or partially purified by ammonium sulfate precipitation. The details of the above procedures have been described previously (Wolfe, Ivler, and Rittenberg, 1954*a*, *b*).

Substrate oxidation was followed by standard Warburg techniques. Biologically formed esters were detected by two methods: trapping experiments in which hydroxylamine was included in the reaction mixture (Lipmann and Tuttle, 1950) and experiments in which hydroxylamine was added at the end of the reaction (Stadtman and Barker, 1950). In both cases the procedure of Stadtman and Barker (1950) was used for preparing the derivatives for chromatographic analysis. Total hydroxamic acids formed were determined by the method of Lipmann and Tuttle (1945).

The hydroxamic acids were chromatographed unidimensionally at room temperature by the ascending technique using Whatman no. 1 filter paper swamped with acetic acid and a butanol-water-acetic acid solution (4:5:1) as the developer (Lugg and Overell, 1948). Free acids were chromatographed in a similar fashion substituting formic acid for acetic acid. In all cases appropriate standards were run on the same paper along with the experimental systems.

Hydroxamic acid standards were prepared from the corresponding ethyl esters (Kornberg and Pricer, 1953). The *alpha-beta* unsaturated and the *beta*-hydroxy capric acids were prepared by the procedures of Thaler and Geist (1939) and Stenhagen (1945), respectively.

EXPERIMENTAL RESULTS

Oxidation of caprate by the crude cell-free extract. Crude cell-free extracts of *P. fluorescens* were tested for their ability to oxidize caprate and acetate in the presence and absence of cofactors known to be required for fatty acid oxidation by other systems. About one micromole of oxygen was consumed per micromole of caprate utilized

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TABLE 1

The effect of ATP and CoA on caprate and acetate oxidation by a crude cell-free extract of Pseudomonas fluorescens

Additions to Reaction Mixture	Substrate	μL O_2 Consumed			
		30 min	60 min	120 min	180 min
None	2 μM caprate	0	0	0	0
ATP	2 μM caprate	2	6	6	18
CoA	2 μM caprate	0	0	4	0
ATP + CoA	2 μM caprate	8	12	23	40
None	2 μM acetate	0	0	0	0
ATP + CoA	2 μM acetate	0	0	0	0

The reaction mixture contained 0.1 ml of the crude cell-free extract (0.62 mg of N), 2 μM of Mg, and 13 μM of GSH, plus 0.1 μM of CoA, 3.3 μM of ATP, and substrate as indicated in a total volume of 2.1 ml. Incubation 30 C; air atmosphere; 0.05 M phosphate buffer, pH 6.1; 0.1 ml 20 per cent KOH in center well for CO_2 absorption. Data corrected for oxygen uptake by enzyme control in absence of substrate.

when ATP,[3] CoA, GSH, and Mg were added to the reaction mixture (table 1). Acetate was not oxidized in the presence of the same supplements. No extra oxygen uptake was observed with caprate as the substrate in the absence of ATP. Oxygen consumption occurred at a reduced rate in the absence of added CoA suggesting residual CoA in the crude cell-free system.

The crude extract was treated with Dowex-1-HCl according to the method of Stadtman, Novelli, and Lipmann (1951) to reduce the level of CoA contamination and retested for CoA dependency. With the Dowex treated preparation oxygen consumption occurred only when CoA and ATP were added together (table 2). The data show an ATP and CoA requirement and suggest an ATP and CoA dependent activation as the initial step in caprate oxidation by this organism.

The inability of the crude extract to oxidize acetate and the observation that only one micromole of oxygen was consumed per micromole of substrate suggested that acetate or AcCoA might accumulate during the reaction. Consequently, at the termination of oxidation the contents of a reaction vessel were esterified, the hydroxamate

[3] The following abbreviations are used: CoA, coenzyme A; AcCoA, acetylcoenzyme A; ATP, adenosine triphosphate; GSH, reduced glutathione; Tris, tris(hydroxymethyl)aminomethane.

TABLE 2

The effect of ATP and CoA on caprate oxidation by a Dowex treated crude cell-free extract of Pseudomonas fluorescens

Additions to Reaction Mixture	μL O_2 Consumed			
	30 min	60 min	120 min	180 min
None	0	0	0	0
ATP	0	0	0	0
CoA	0	0	0	0
ATP + CoA	12	22	39	45

The reaction mixture contained 0.1 ml of extract (0.06 mg of N), 2 μM of caprate, 2 μM of Mg, and 13 μM of GSH, plus 0.1 μM of CoA and 3.3 μM of ATP as indicated, in a total volume of 3.1 ml. Incubation at 30 C; air atmosphere; 0.05 M phosphate buffer, pH 6.1; 0.1 ml 20 per cent KOH in center well for CO_2 absorption. Data corrected for oxygen uptake by enzyme control in absence of substrate.

derivatives prepared, and the mixture chromatographed. The chromatograms showed a spot with an R_f of about 0.63 corresponding to the acethydroxamate control, 0.62–0.65. No spot corresponding to caprylhydroxamate was detected as should be expected. This may have been due to the small amount of substrate initially present.

Caprate oxidation by the partially purified extract. The various fractions obtained by ammonium sulfate precipitation of the crude extract were checked for their ability to oxidize caprate. The oxidizing system was located in the material precipitating between 40 and 60 per cent ammonium sulfate saturation (S_{40-60}). This fraction, when supplemented with ATP, CoA, GSH, and Mg, consumed only 0.5 micromole of oxygen per micromole of caprate, after a lag of about 100 minutes. With this system, chromatographic and chemical tests failed to reveal the presence of acetate and keto acids but showed an as yet unidentified compound with an R_f of 0.22 detected as the free acid. *Alpha-beta* unsaturated and *beta*-hydroxy caprates, either of which could be formed from caprate with the consumption of 0.5 micromole of oxygen, chromatographed as the free acids gave R_f's of 0.24 and 0.26, respectively.

In the presence of fluoride (50 μM/ml) the lag period in the oxidation of caprate was decreased. Since fluoride is known to complex with magne-

sium (Warburg and Christian, 1941), the effect of the latter on the caprate oxidizing system was investigated. Five ml of the S_{40-60} fraction were dialyzed against two, two liter portions of cold (4 C) distilled water for twelve hours each. The dialyzed preparation was then checked for its ability to oxidize caprate in the presence of ATP, CoA, and GSH, with and without magnesium (1 μM/ml). The data show (figure 1) an immediate oxygen uptake in the absence of added magnesium with a total consumption of about a half micromole per micromole of caprate. The total oxygen uptake was the same in the presence of magnesium, but the same lag observed with the undialyzed preparation occurred. It is apparent that magnesium at the level used inhibits caprate oxidation by the partially purified system. A similar effect has been noted by Newcomb and Stumpf (1952) in palmitate oxidation by an enzyme preparation from peanut cotyledons.

Trapping of active caprate. Qualitative trapping experiments were run both in the presence and absence of oxygen. An undialyzed S_{40-60} fraction was used, and hydroxylamine was included in the reaction mixture. At the end of the reaction period the mixture was deproteinized, concentrated, and chromatographed. Both the aerobic and anaerobic flasks gave spots with an R_f of 0.96–0.98 that did not arise from the control flasks and which corresponded to the caprate standard, R_f 0.95–0.98. Similar experiments showed that the S_{40-60} fraction was also capable of activating acetate.

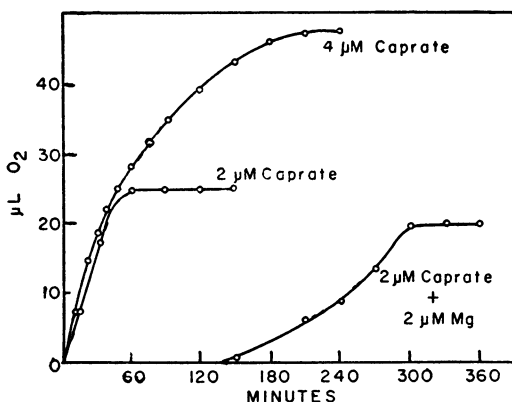

Figure 1. The effect of magnesium on caprate oxidation by a dialyzed, ammonium sulfate precipitated fraction from *Pseudomonas fluorescens*.

The reaction mixtures contained 0.1 ml dialyzed S_{40-60} fraction (0.073 mg N), 0.1 μM of CoA, 13 μM of GSH, 3.3 μM of ATP, 5 μM K_2HPO_4 plus 2 μM Mg where indicated in a total volume of 2.1 ml. Incubation 30 C, air atmosphere, 0.01 M Tris buffer, pH 7.4. 0.1 ml 20 per cent KOH in center well for CO_2 absorption. Corrected for oxygen uptake by enzyme control in absence of substrate.

TABLE 3

Trapping of activated caprate with hydroxylamine

Additions to Reaction Mixture	Total Hydroxamate Formed	R_f Values of Hydroxamate*
	μM	
ATP + CoA	1.0; 1.3	0.97; 0.97
ATP + CoA†	2.1	0.96
CoA	0.5	—
GSH + Mg	0.3	—
None	0 to 0.2	—

* R_f of the capric hydroxamate standard is 0.95–0.98.

† Reaction run in air atmosphere.

The reaction mixture contained 1.0 ml of the S_{40-60} fraction (0.72 mg of N), 20 μM of caprate, 100 μM of NH_2OH, 2 μM of Mg, 5 μM of K_2HPO_4, 82 μM of GSH and where indicated, 0.6 μM of CoA and 13.2 μM of ATP in a total volume of 5.0 ml. Incubation 30 C for 90 minutes; nitrogen atmosphere, Tris buffer pH 7.4.

For the quantitative determination of biologically formed hydroxamates reaction vessels were set up as shown in table 3. Determinations were run on 1 ml aliquots of the reaction mixture after 90 minutes' incubation; the remainder of the reaction mixture was concentrated and chromatographed. Appreciable quantities of hydroxamic acids were formed aerobically and anaerobically if ATP and CoA were present. In the absence of these cofactors essentially no hydroxamic acid was detected.

DISCUSSION

From the data presented it is concluded that the mechanism of caprate oxidation by *P. fluorescens* is a *beta* oxidation of an activated caprate. The pathway parallels that of fatty acid oxidation in mammalian tissues (see for example Mahler, 1953; Lynen, 1954) and in *Clostridium kluyveri* (Barker, 1951; Stadtman, 1953). This conclusion is substantiated by the following experimental evidence: (1) The demonstration of an ATP, CoA requirement for caprate oxidation by the cell-free system. (2) The trapping of an active ester of caprate under anaerobic condi-

tions. (3) The demonstration of acetate formation from caprate with the consumption of only one micromole of oxygen per micromole of caprate and evidence for either *beta*-hydroxy or *alpha-beta* unsaturated caprate formation with an uptake of a half micromole of oxygen per micromole of caprate. Because the system employed could activate acetate, it was not possible to distinguish between acetate and active acetate as the direct product of cleavage. Likewise, the compound formed when only a half micromole of oxygen was consumed was demonstrated as the free acid. However, previous work has ruled out the unactivated compounds as direct intermediates in the oxidation (Silliker and Rittenberg, 1952; Waltman and Rittenberg, 1954), and it can be assumed that the activated compounds are the true intermediates.

The failure to detect caprylate as well as acetate in the reaction mixture when one micromole of oxygen was consumed per micromole of caprate may have been due to the insensitivity of the chromatographic method. However, if caprylCoA was formed by a thiolytic cleavage, one must account for the lack of its further oxidation as evidenced by the oxygen uptake data, since it has been shown that the same preparation oxidizes caprylate, again with the uptake of one micromole of oxygen per micromole of substrate (Ivler, 1954). Lack of further oxidation might be explained by a limiting quantity of some cofactor in the hydrogen transport system. This explanation is not satisfactory, however, since doubling the level of substrate doubles the oxygen consumption at a constant level of ATP and CoA. An alternate possibility is that the cleavage enzyme is lacking in the crude preparation and that the acetate detected was formed nonenzymatically from the activated ketocaprate during the manipulations required for esterification and hydroxamate formation.

SUMMARY

A cell-free extract was obtained from *Pseudomonas fluorescens* that oxidized caprate, consuming one micromole of oxygen per micromole of substrate. Adenosine triphosphate and coenzyme A were required for the oxidation, and acetate was found at the end of the reaction. The extract after partial purification by ammonium sulfate fractionation consumed only a half micromole of oxygen per micromole of caprate. Magnesium ion at one micromole per ml temporarily inhibited the oxidation. An unidentified compound accumulated in the reaction that had an R_f similar to those for *beta*-hydroxy and *alpha-beta* unsaturated caprate. An active caprate was trapped under anaerobic conditions. It is concluded that the mechanism of caprate oxidation by *P. fluorescens* is a *beta* oxidation of an activated caprate.

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