STUDIES ON THE AEROBIC OXIDATION OF FATTY ACIDS BY BACTERIA

IV. The Effect of 2,4,6-Trichlorophenol on the Oxidation of Caprate and its Derivatives by *Serratia marcescens*[1]

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It was previously shown that adaptation of *Serratia marcescens* to the oxidation of capric acid simultaneously adapted this organism to the oxidation of α-β unsaturated, β-hydroxy, and β-ketocapric acids (Silliker and Rittenberg, 1951*a*, *b*). These data were interpreted as indicating that the mentioned derivatives are intermediates or are closely related to intermediates in the oxidation of capric acid. More recently it has been shown that the α-β unsaturated, β-hydroxy, and β-keto derivatives, as activated forms, are intermediates in fatty acid oxidation by animal systems (Lynen and Ochoa, 1953; Mahler, 1953; and others).

This paper deals with the effects of a series of inhibitors on the oxidation of the above mentioned compounds. It was hoped that a compound could be found that would block fatty acid oxidation at an early stage and thus make the identification of accumulated intermediates possible.

MATERIALS AND METHODS

S. marcescens (Alphin strain) was the test organism. It was maintained on a medium of the following composition: K_2HPO_4, 5.0 g; KH_2PO_4, 2.0 g; NH_4NO_3, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; caprate, 0.01 M; agar, 20 g; distilled water, 1000 ml; pH 7.0. Cells were grown on the above medium for 48 hours at 37 C, harvested and washed four times with 0.04 M phosphate buffer (pH 7.0), and suspended in the same buffer to give a turbidity reading of 350 at 540 mμ (Klett-Summerson photoelectric colorimeter).

The cell suspensions were used in oxidation studies following standard Warburg procedures (Umbreit *et al.*, 1949). The reaction flasks contained 0.5 ml of substrate in one side arm and, when used, 1 ml of inhibitor (in phosphate buffer) in the other side arm. One ml of cell suspension plus sufficient buffer to give a total volume of 3 ml was added to the main well. The center well contained 0.1 ml of 20 per cent KOH.

In most cases the substrate and inhibitor were tipped in simultaneously from the side arms at zero time. Oxygen uptake was then followed for 3 to 4 hours at 30 C in an air atmosphere. Autorespiratory values were determined in the presence and absence of inhibitor. The oxidative data presented are corrected for these values.

RESULTS

A total of thirteen inhibitors was screened. Four patterns were observed: no effect; inhibition of rate; inhibition of rate and total oxygen uptake; and a differential effect. Table 1 records representative data obtained with inhibitors giving the first three effects.

Sodium fluoride gave results typical of the "no effect" group. At concentrations of up to 0.01 M this compound did not adversely affect either the amount or rate of oxidation of caprate and its β-oxidation derivatives. Arsenate, up to 0.01 M, and streptomycin, up to 0.67 mg per ml, likewise had no effect.

Terramycin is representative of the compounds that inhibited the rate of oxidation only. At the three concentrations of the antibiotic tested there was no inhibition of the total oxygen uptake on the fatty acids, in fact a slightly increased uptake was found in some cases. The rate of oxidation, however, was reduced although not in proportion to the concentration of antibiotic used.

Similar results were obtained with malonate

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TABLE 1

The effect of various inhibitors on the extent and rate of oxidation of caprate and its derivatives by Serratia marcescens

Inhibitor	Concentration	Oxygen Uptake (% of control)*				Rate (% of control)*			
		C_{10}	$\Delta_{2,3}$ C_{10}	β-Hydroxy C_{10}	β-Keto C_{10}	C_{10}	$\Delta_{2,3}$ C_{10}	β-Hydroxy C_{10}	β-Keto C_{10}
Sodium fluoride	0.001 M	102	108	89	106	100	104	112	123
	0.01 M	109	109	101	95	109	100	108	91
Terramycin·HCl	0.15 mg/ml	110	109	125	106	86	70	87	103
	0.30 mg/ml	>100	>100	>100	98	72	56	81	95
	0.60 mg/ml	103	>100	100	114	72	64	72	65
Iodoacetate	0.0001 M	84	95	90	—	50	58	52	—
	0.001 M	52	42	47	—	38	37	33	—
	0.01 M	33	22	32	30	14	20	25	25

* Oxygen uptake and rate of oxidation in absence of inhibitor used as control (100%). All values corrected for autorespiration in presence of inhibitor.

and fluoroacetate. The former decreased the rate of oxidation at a concentration of 0.02 M but not at 0.01 M. The latter, at a level of 0.00067 M, depressed the rate of oxidation approximately 40 per cent; higher concentrations, up to 0.01 M, did not accentuate the inhibition.

Iodoacetate influenced both the rate and extent of oxidation. As its concentration was increased from 0.0001 M to 0.01 M a progressive increase in degree of inhibition was observed. Arsenite, azide, cyanide, 2,4-dinitrophenol (DNP), and hydroxylamine acted qualitatively the same although they showed quantitative differences.

All the compounds mentioned so far, whatever

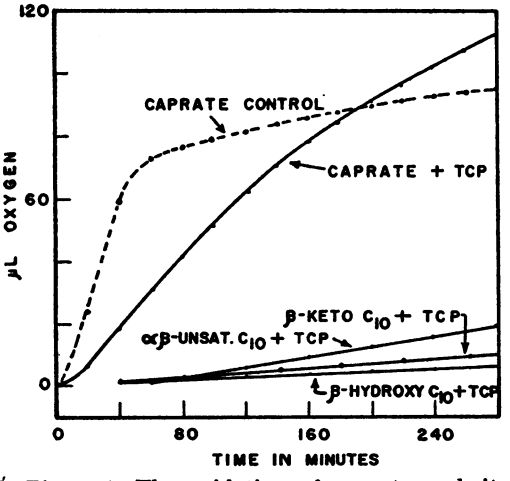

Figure 1. The oxidation of caprate and its derivatives by *Serratia marcescens* in the presence of 0.00067 M 2,4,6-trichlorophenol. 0.5 μM substrate per flask. All values corrected for autorespiration in presence of inhibitor.

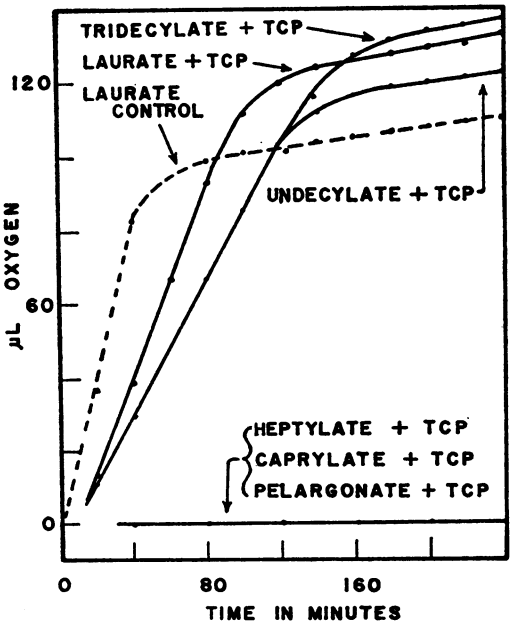

Figure 2. Oxidation of various fatty acids by *Serratia marcescens* in the presence of 0.00067 M 2,4,6-trichlorophenol. 0.5 μM substrate per flask. All values corrected for autorespiration in presence of inhibitor.

their effects, influenced the oxidation of caprate and its derivatives essentially to an equal degree. 2,4,6-Trichlorophenol (TCP), however, acted in a differential manner. At a concentration of 0.00067 M trichlorophenol the oxygen uptake on caprate was increased although the rate of oxidation was lowered (figure 1). At this same concentration of trichlorophenol, the oxidation of the three caprate derivatives was completely or almost completely suppressed (figure 1). Similar effects were obtained when the concentration of trichlorophenol employed was halved or doubled.

A concentration of 0.00067 M trichlorophenol also completely suppressed the oxidation of heptylate (C_7), caprylate (C_8), and pelargonate (C_9) while the same concentration enhanced total oxygen uptake (at lower rate) with undecylate (C_{11}), laurate (C_{12}), and tridecylate (C_{13}) (figure 2). The effect of trichlorophenol on hexanoate oxidation was not checked at the 0.00067 M trichlorophenol level. However, complete inhibition of the oxidation of this compound was obtained with 0.001 M trichlorophenol. Thus inhibition by trichlorophenol was related to the chain length of the substrate.

DISCUSSION

Although none of the inhibitors tested caused the accumulation of an intermediate in the early stages of fatty acid oxidation, one of the compounds used, trichlorophenol, had an effect which may make it valuable for further investigations of fatty acid metabolism. Since trichlorophenol completely blocks the oxidation of the β-oxidation derivatives of caprate as well as the oxidation of shorter chain length fatty acids (C_6–C_9) under conditions where the oxidation of caprate and longer chain length fatty acids (C_{11}–C_{13}) is not inhibited, the metabolism of these two groups of compounds must involve at least one different enzyme.

It would appear from the data obtained that trichlorophenol is inhibiting an activating enzyme(s). If the oxidation of an activated fatty acid was the step being blocked, only a partial oxidation of the higher fatty acids should have occurred in the presence of this inhibitor. On this basis it can be postulated, that for *S. marcescens* at least, caprate and longer chain length fatty acids are activated by a different enzyme(s) from that which activates the caprate derivatives and the lower fatty acids.

Recent work has established that an activation of the fatty acid molecule is a prerequisite for oxidation; this has been shown for animal systems (Drysdale and Lardy, 1952; Green and Mii, 1953; Kornberg and Pricer, 1953; Mahler, 1953; Mahler *et al.*, 1953) and bacterial systems (Barker, 1951; Stadtman, 1953; Ivler, 1954). The activating enzymes from animal systems are somewhat specific as to the chain length of the substrate activated. Mahler (1953), for example, found that the enzyme that activates C_4 to C_{10} fatty acids, caused a slow activation of the C_{11} but did not activate the C_{12} acid.

Trichlorophenol obviously influenced other enzymatic processes as well as is evidenced by the increased oxygen uptake in its presence when caprate and the higher fatty acids were substrates. This effect could be explained by assuming that trichlorophenol also acts like the related phenol, 2,4-dinitrophenol, in preventing adenosine triphosphate formation. It should be mentioned that trichlorophenol has been used as an inhibitor of bacterial metabolism in at least one previous investigation. Shibata and Yamaguchi (1948) found that this compound caused a marked inhibition of succinate oxidation by *Escherichia coli*.

Considering the uncertainties inherent in inhibition studies on whole cells, the data obtained with the other inhibitors used in this study are consistent with present knowledge of fatty acid oxidation. Malonate, fluoroacetate, and terramycin are all known to inhibit at various points in the tricarboxylic acid cycle (Krebs, 1948–1949; Buffa *et al.*, 1951; Hobby, 1953), and their depression of the rate of oxidation of caprate is explainable on this basis. Among the compounds that inhibited both the rate and extent of oxidation are some known to affect either adenosine triphosphate formation (2,4-dinitrophenol, azide) or else inhibit sulfhydryl groups (iodoacetate, arsenite). Since both adenosine triphosphate and coenzyme A are required for fatty acid oxidation, the observed inhibitions are to be expected.

Dinitrophenol may have, in addition, effects similar to trichlorophenol. In previous work (Silliker, 1950; Silliker and Rittenberg, 1952) it was found that dinitrophenol influenced fatty acid oxidation by *S. marcescens* in a complicated manner. The amount of inhibition, if any, was dependent on the chain length of the fatty acid,

the pH at which the oxidation was carried out, and on whether adapted or nonadapted cells were used. Some of these data can now be explained by assuming that dinitrophenol acts differentially on activating enzymes.

The lack of inhibition by fluoride, arsenate, and streptomycin could mean either that the enzymes inhibited by these compounds are not involved in fatty acid oxidation or else that owing to various experimental conditions these compounds were not present at inhibitory levels within the cells. Therefore no inferences should be drawn from these negative results.

SUMMARY

Thirteen metabolic inhibitors have been tested for their effects on the oxidation of capric acid and its derivatives, α-β unsaturated, β-hydroxy and β-ketocapric acids, by resting cells of *Serratia marcescens*. These inhibitors fell into four groups: those without any effect; those depressing the rate of oxidation only; those inhibiting both the rate and extent of oxidation; and a single compound, 2,4,6-trichlorophenol, which showed differential inhibitory effects depending on the chain length of the substrate. At the proper concentration the latter inhibitor prevented the oxidation of the caprate derivatives and shorter chain length fatty acids but did not inhibit the oxidation of caprate and longer chain fatty acids. It was suggested that trichlorophenol acted by blocking the activating enzyme(s) involved.

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