STUDIES ON THE AEROBIC OXIDATION OF FATTY ACIDS BY BACTERIA

III. The Effect of 2,4-Dinitrophenol on the Oxidation of Fatty Acids by Serratia marcescens[1]

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It has been shown that glucose grown *Serratia marcescens* oxidizes a number of normal fatty acids only after a lag period; however, cells harvested from a medium containing a fatty acid as the sole carbon source, as well as cells exposed to an oxidizable acid, show no significant lag period in the oxidation of any of the acids (Silliker and Rittenberg, 1951*a*,*b*). These data indicate that the oxidation of such substrates is catalyzed by adaptive enzymes, and applying the concept of simultaneous adaptation (Stanier, 1947) it was postulated that a single group of enzymes functioning in a repeating sequence is involved in the oxidation of a wide variety of fatty acids.

The present paper is concerned with the effect of 2,4-dinitrophenol and sodium azide on the oxidation of fatty acids by *S. marcescens*. The study was initiated because of the following considerations: these compounds prevent the formation of adaptive enzymes (Monod, 1944; Reiner, 1946; Spiegelman, 1947), and if they prevent the oxidation of fatty acids by glucose grown cells (unadapted), the postulate of an adaptive enzyme system for fatty acid oxidation in *S. marcescens* is strengthened. Also, 2,4-dinitrophenol and sodium azide are known to prevent oxidative assimilation (Clifton, 1937). Thus, by studying the relationship between the amount of oxygen consumed and the amount of substrate oxidized in the presence of appropriate concentrations of the cell poisons it should be possible to determine the extent to which the various fatty acids are oxidized, with data uncomplicated by oxidative assimilation.

EXPERIMENTAL METHODS

The methods employed were similar to those previously described (Silliker and Rittenberg, 1951*a*,*b*). Unadapted cells were harvested from glucose mineral salts medium, while adapted cells were obtained either by exposure of glucose grown cells to specific acids in the Warburg vessels or by growing cells on media containing fatty acids as the sole source of carbon.

Uptake of oxygen was determined manometrically in the usual manner (Um-

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breit *et al.*, 1949). In determining net oxygen uptake due to substrate oxidation, it was necessary to consider the effect of endogenous respiration on the observed gas uptake in the presence of substrate. Barker (1936), Clifton (1937), Doudoroff (1940), and Wiame and Doudoroff (1951) have found that when oxidation of substrate proceeds at a rapid rate, the amount of endogenous respiration is negligible. Where substrate is utilized at a slow rate, oxygen uptake due to endogenous respiration becomes a significant factor, and appropriate corrections must be made; this usually involves subtracting endogenous uptake of oxygen from that observed in the presence of substrate. Norris *et al.* (1949), using *Pseudomonas aeruginosa*, and Cochrane and Gibbs (1951), using *Streptomyces coelicolor*, have encountered systems in which endogenous respiration proceeds at the normal rate even when oxidation of substrate is rapid. In the present study it was found that when fatty acids were rapidly oxidized, endogenous respiration was negligible. In such cases, net oxygen uptake due to substrate oxidation was determined by extension of the steepest part of the oxidation curve to meet the projection of the curve after the autorespiratory oxygen uptake level had been reached. With this procedure the values obtained in different experiments were comparable, and oxygen uptake per unit of substrate was nearly constant over the range of concentrations tested. Where the rate of oxidation was very slow, the total oxygen uptake in the presence of substrate was corrected by subtracting autorespiratory uptake. Where oxidation of substrate was slow, it was difficult to obtain closely reproducible values.

The 2,4-dinitrophenol and sodium azide were dissolved in appropriate concentrations in the same buffer solution (M/20 phosphate) used for preparing the cell suspensions and substrates. In all cases the cell poisons and the substrates were introduced simultaneously from the side arm into the main well of the Warburg vessel containing the cells.

RESULTS

Effect of 2,4-dinitrophenol on the oxidation of fatty acids at pH 7.0. Table 1 summarizes the results obtained when resting cellular suspensions of *S. marcescens* were exposed to fatty acids in the presence and in the absence of 2,4-dinitrophenol. In the absence of the cell poison, oxygen uptake was always below the theoretical amount required for complete oxidation. It is apparent at once that oxidative assimilation occurs with all the acids tested, provided that no intermediate products accumulate in the oxidation of these substrates. With this assumption, the data seem to indicate that the amount of assimilation increases with increasing molecular weight of the fatty acids up to C_8 as is shown by the relatively higher oxygen uptake on the C_2, C_6, and C_7 acids. The failure of *S. marcescens* to grow on acetate as the sole source of carbon may be related to the almost complete oxidation and lack of oxidative assimilation of this compound.

In preliminary experiments with capric acid oxidation, it was found that the cellular suspensions gave maximum oxygen consumption at 2,4-dinitrophenol levels of 0.0005 to 0.00075 M. A level of 0.001 M 2,4-dinitrophenol gave slightly

lower values. Accordingly, most of the experiments were conducted with 0.0005 or 0.00075 M 2,4-dinitrophenol, although these concentrations are not necessarily optimum for all the fatty acids.

Table 1 shows that three distinct patterns were observed with respect to the oxidation of fatty acids in the presence of 2,4-dinitrophenol: (1) direct inhibition of oxidation both in adapted and unadapted cells (acetic, caproic (C_6), and heptylic (C_7) acids); (2) inhibition of oxidation in unadapted but not in adapted

TABLE 1

Effect of 2,4-dinitrophenol (DNP) on the oxidation of fatty acids by unadapted and adapted cell suspensions of Serratia marcescens

SUBSTRATE	OXYGEN CONSUMPTION IN PER CENT OF THEORETICAL FOR COMPLETE OXIDATION†			
	Unadapted cells		Adapted‡ cells	
	No DNP	0.00075 M DNP	No DNP	0.00075 M DNP
Acetate	74	6*	84	17*
Caproate	73	0*	68	0
Heptylate	67	0*	53	11*
Caprylate	58	20*	53	86
Pelargonate	52	8*	63	85
Caprate	56	99	55	84
Undecylate	51	18*	53	82
Laurate	62	105	59	90
Tridecylate	53	11*	50	81
Myristate	62	>47*	57	80

* Rate of oxidation very slow; accurate values difficult to determine.

† Values corrected for autorespiration and represent averages from many determinations.

‡ All cells were adapted by growth on caprate except for acetate oxidation where exposure to acetate was used for adaptation.

Experiments conducted at 30 C in air atmosphere with 10 per cent KOH in center well to absorb carbon dioxide. 0.4 to 2.0 micromoles of substrate per flask, depending on particular acid used.

cells (caprylic (C_8), pelargonic (C_9), undecylic (C_{11}), and tridecylic (C_{13}) acids); and (3) no inhibition of oxidation either in adapted or in unadapted cells (capric (C_{10}), lauric (C_{12}), and myristic (C_{14}) acids). It will be noted that in some cases the degree of inhibition was incomplete, viz., 17 per cent of complete oxidation of acetate by adapted cells in the presence of 2,4-dinitrophenol and 11 per cent oxidation of heptylate by adapted cells. In these and in similar cases of apparent incomplete inhibition the actual rate of oxygen uptake was very low; here we may be dealing with a slight stimulation of endogenous metabolism or alternatively with a concentration of 2,4-dinitrophenol which is only partially effective.

Of particular interest is the oxidation of capric, lauric, and myristic acids in the presence of 2,4-dinitrophenol. As has been previously mentioned, 2,4-dinitrophenol has been shown to be an inhibitor of enzymatic adaptation. Yet in its

presence unadapted cells oxidized these substrates to completion with a characteristic lag period suggestive of enzymatic adaptation, even though the rate of oxidation by unadapted cells in the presence of 2,4-dinitrophenol was considerably lower than the rate with adapted cells and 2,4-dinitrophenol. The situation was investigated in some detail with respect to caprate oxidation. It was found that if unadapted cells were allowed to oxidize capric acid to completion in the presence of 2,4-dinitrophenol and then were exposed to more caprate (poured from the side arm of the Warburg vessel), the oxidation once again proceeded only after a lag period. Such cells also showed a lag period in the oxidation of other fatty acids. It will be recalled that cells allowed to oxidize fatty acids in the absence of 2,4-dinitrophenol subsequently oxidize caprate and all the other acids without a lag period (Silliker and Rittenberg, 1951*b*). It appears, therefore, that while the unadapted cells are capable of caprate oxidation in the presence of 2,4-dinitrophenol, the process of adaptation to other acids by such cells is blocked; in fact, even adaptation to capric acid is prevented if elimination of the lag period is to be considered the criterion for adaptation.

The presence of 2,4-dinitrophenol increased the uptake of oxygen with adapted cells on the C_8 to C_{14} substrates from between 50 to 60 per cent to between 80 to 90 per cent of theoretical. Whether the 10 to 20 per cent deficit in oxygen consumption represents incomplete blockage of oxidative assimilation by 2,4-dinitrophenol or alternately the accumulation of some incompletely oxidized end product cannot be decided conclusively from the data. However, unadapted cells in the presence of 2,4-dinitrophenol do consume completely caprate and laurate, and it is unlikely that the oxidative pathways differ in the two types of cells. It is highly probable, therefore, that in the normal metabolism of the C_8 to C_{14} acids the fraction that is not oxidatively assimilated is oxidized completely to CO_2 and water.

Neither heptylate nor caproate was appreciably oxidized in the presence of 2,4-dinitrophenol at pH 7.0; therefore, indications as to the extent of oxidation of these acids can be obtained only from the data determined in the absence of 2,4-dinitrophenol. These results show at least a 73 per cent oxidation of caproate and 67 per cent oxidation of heptylate. Since work with the higher acids indicated that in the absence of 2,4-dinitrophenol 30 to 40 per cent of the substrate is assimilated, it is again probable that no unoxidized fragments are formed during the metabolism of the C_6 and C_7 acids. While acetate oxidation was blocked by 2,4-dinitrophenol at pH 7.0, data to be presented in the next section indicate that the organism carries out a complete oxidation of acetic acid.

The effect of 2,4-dinitrophenol on the oxidation of fatty acids at pH 8.0. The effectiveness of 2,4-dinitrophenol as an inhibitor of oxidative assimilation is related to the hydrogen ion concentration (Doudoroff, 1940). In the preceding section the possibility suggested itself that 2,4-dinitrophenol might exert at least two distinct effects on fatty acid oxidation, i.e., direct blockage of the activity of the enzymes catalyzing oxidation and inhibition of enzymatic adaptation. Accordingly, experiments were carried out to determine the effect of 2,4-dinitrophenol on acetate and caprate oxidation at pH 8.0. Data summarized in

table 2 indicate that whereas acetate oxidation and adaptation to its oxidation are blocked at pH 7.0 by 2,4-dinitrophenol, neither process is inhibited by the compound at pH 8.0. On the other hand, capric acid is readily oxidized by both adapted and unadapted cells at either pH in the presence of 0.00075 M 2,4-dinitrophenol. Of significance is the fact that whereas 2,4-dinitrophenol does not inhibit either adaptation or oxidation of acetate at the higher pH, it does appear to inhibit oxidative assimilation, since oxygen consumption by unadapted cells increases from 74 to 93 per cent of the theoretical oxygen uptake required for complete oxidation.

TABLE 2

The effect of pH on the oxidation of acetic and capric acids by Serratia marcescens in the presence and in the absence of 2,4-dinitrophenol (DNP)

SUBSTRATE	EXPERIMENTAL CONDITIONS	PER CENT OF THEORETICAL OXYGEN UPTAKE*
Acetate	pH 7.0, unadapted cells	74
	pH 7.0, unadapted cells, 0.0005 M DNP	6†
	pH 7.0, C_2-exposed cells	84
	pH 7.0, C_2-exposed cells, 0.0005 M DNP	17†
	pH 8.0, unadapted cells	78
	pH 8.0, unadapted cells, 0.00075 M DNP	93
	pH 8.0, C_{10}-grown cells, 0.00075 M DNP	62
Caprate	pH 7.0, unadapted cells	56
	pH 7.0, unadapted cells, 0.00075 M DNP	99
	pH 7.0, C_{10}-exposed cells	55
	pH 7.0, C_{10}-exposed cells, 0.00075 M DNP	84
	pH 8.0, unadapted cells, 0.00075 M DNP	98
	pH 8.0, C_{10}-grown cells, 0.00075 M DNP	74

* Values corrected for autorespiration.

† Rate of oxidation very slow; accurate values difficult to determine. Acetic acid flask concentration, 0.001 M; capric acid flask concentration, 0.0002 M. All experiments conducted in an air atmosphere with 10 per cent KOH in center well to absorb carbon dioxide. 30 C, M/20 phosphate buffer.

It is of interest to note that neither capric nor acetic acids are oxidized to completion by adapted cells at pH 8.0 in the presence of 2,4-dinitrophenol, but unadapted cells show oxygen uptake, indicating complete oxidation to carbon dioxide and water under the same conditions. A similar finding is shown in the preceding section. At pH 7.0 in the presence of 2,4-dinitrophenol, capric and lauric acids were completely oxidized by unadapted cells (99 per cent and 105 per cent) but showed only 84 per cent and 90 per cent of the theoretical oxygen consumption with C_{10}-grown organisms. This suggests that a higher concentration of 2,4-dinitrophenol may be required to inhibit oxidative assimilation in adapted cells. Alternatively, the relatively slower oxidation carried out by unadapted cells in the presence of 2,4-dinitrophenol may result in a significant error due to the effect of endogenous respiration. Wiame and Doudoroff (1951) have reported that whereas endogenous respiration is normally inhibited during the oxidation

of exogenous substrate, in the presence of 2,4-dinitrophenol the endogenous respiration proceeds at approximately the normal rate. While our experiments suggest that endogenous respiration is suppressed during rapid substrate oxidation, even in the presence of 2,4-dinitrophenol, the relatively slower oxidation by unadapted cells may account for the observed quantitative differences between the extent of assimilation in adapted and unadapted organisms.

In the presence of 2,4-dinitrophenol at pH 7.0 adapted cells consumed only 84 per cent and 85 per cent of the amount of oxygen required for complete oxidation of caprate and pelargonate. Analysis of these data indicates that if the uptakes below theoretical were due to incomplete oxidation the unoxidized fragments should correspond closely to acetic acid. Since acetic acid is not oxidized under these conditions, it seemed possible that it might accumulate. To check this possibility experiments were conducted in which capric and pelargonic acids were oxidized by adapted cells at pH 7.0 in the presence of 2,4-dinitrophenol. When the autorespiratory level was reached, alkaline buffer was poured from the side arm, raising the pH in the flask to 8.0. With neither acid was there any further oxygen uptake above the autorespiratory level after the pH was raised. If acetic acid had accumulated at pH 7.0, one would have expected further oxygen uptake after the pH had been raised, since our data show that acetate is oxidized at the higher pH under the conditions of the experiment. The results indicate that acetate does not accumulate in either caprate or pelargonate oxidation at pH 7.0 in the presence of 2,4-dinitrophenol and strengthen the conclusion that the apparent incomplete oxidation of the C_8 to C_{14} acids is due to incomplete inhibition of oxidative assimilation.

The effect of sodium azide on the oxidation of fatty acids. Effects similar to those produced by 0.00075 M 2,4-dinitrophenol were produced by 0.06 M sodium azide, but the over-all oxygen uptake was not as great as in the presence of the phenolic compound. Azide inhibited acetate oxidation at pH 7.0 to the same extent as did 2,4-dinitrophenol. Caproate and heptylate oxidation, as well as that of the C_{11} through C_{14} acids, was not studied in the presence of azide. Sodium azide increased oxygen uptake on caprylic, pelargonic, and capric acids when adapted cells were used but not to the same extent as did 2,4-dinitrophenol. With unadapted cells, azide had no inhibitory effect on caprate oxidation but inhibited pelargonate oxidation to the same extent as did 2,4-dinitrophenol. No data were obtained for unadapted cells in the presence of azide and caprylate.

DISCUSSION

2,4-Dinitrophenol exerts its influence on cellular metabolism by inhibiting inorganic phosphate utilization during oxidative processes, thus preventing the use of energy provided by respiration by blocking the formation of high energy phosphate bonds (Loomis and Lipmann, 1948). With respect to the effect of 2,4-dinitrophenol on fatty acid oxidation, the reports are conflicting. Clifton (1946) showed that both acetate and butyrate are oxidized by *Pseudomonas calco-acetica* in the presence of 2,4-dinitrophenol; Fantl *et al.* (1951) found that 2,4-dinitrophenol enhanced the respiration of rat liver metabolizing caprylic

acid, the proportion of acetoacetic acid to beta hydroxy butyric acid in the products of oxidation being much greater in the presence than in the absence of 2,4-dinitrophenol. Judah and Williams-Ashman (1951) found only a slight inhibition of beta hydroxy butyric acid oxidation by the cyclophorase system. Cross *et al.* (1949), in their studies on the cyclophorase system, showed that the oxidation of fatty acids requires a "sparking reaction" consisting of the oxidation of a small amount of one of the members of the tricarboxylic acid cycle. This reaction involves the uptake of inorganic phosphate. In the presence of 2,4-dinitrophenol the oxidation of the tricarboxylic acid cycle members is unimpaired, but the phosphate uptake usually accompanying this oxidation is inhibited. Under these conditions no oxidation of acetate, butyrate, or beta keto caprylic acid occurred. Stoppani (1949) reported the inhibition of acetate oxidation and phosphate uptake in yeast in the presence of 2,4-dinitrophenol, and Judah and Williams-Ashman (1951) confirmed the inhibition of acetate oxidation and phosphate uptake in the cyclophorase system.

In the present study, 2,4-dinitrophenol directly inhibited the oxidation of acetic, caproic, and heptylic acids. Under conditions where the oxidation of these acids was blocked, experimental evidence was obtained for the complete oxidation of the C_8 through C_{14} fatty acids. Hence, it is unlikely that either acetic, caproic, or heptylic acids are direct intermediates in the oxidation of the higher acids. Since capric and lauric acids are completely oxidized by unadapted cells in the presence of 2,4-dinitrophenol, the failure of unadapted cells to oxidize caprylic acid under the same conditions leads to the conclusion that the 10 and 12 carbon acids are dissimilated through a pathway not involving the 8 carbon acid. Since beta oxidation would involve caprylic, caproic, butyric, and acetic acids as intermediates in the oxidation of higher even chain fatty acids and heptylic and acetic acids as intermediates in the dissimilation of higher odd chain acids, these findings, together with the inability of both living and dried cells to use butyric acid, strongly rule against classical beta oxidation as the pathway of fatty acid oxidation.

The possibility exists, of course, that "active" forms of the lower acids are intermediates and that the production of these molecular species from normal fatty acids is blocked by 2,4-dinitrophenol. If this were the case, then the essential features of beta oxidation would remain, but the chemical nature of the intermediates would be somewhat different than those that have been predicted on the basis of Knoop's theory. The results, however, might be explained on the basis of a multiple alternate oxidation mechanism; such a pathway would allow complete oxidation of a fatty acid without lower normal acids occurring as intermediates. It has been found, in fact, that dried cells of *S. marcescens* carry out incomplete oxidations of fatty acids, the nature of which suggests multiple alternate rather than beta oxidation (Silliker, 1951).

Monod (1944), Reiner (1946), and Spiegelman (1947) have shown that 2,4-dinitrophenol and azide inhibit the formation of certain adaptive enzymes in microorganisms. If this property of 2,4-dinitrophenol held for all adaptive enzymes, one would conclude that the C_8, C_9, C_{11}, and C_{13}, and perhaps the C_3,

acids are oxidized through adaptive enzymes, while the C_{10}, C_{12}, and C_{14} acids are attacked through constitutive enzymes. The type of attack on caproic and heptylic (and perhaps on acetic) might be either constitutive or adaptive since 2,4-dinitrophenol apparently interferes directly with some oxidative reaction. On the basis of the experiments with simultaneous adaptation (Silliker and Rittenberg, 1951*b*), it appears unlikely that some of the acids are attacked adaptively and others constitutively. Exposure of cells to any of the fatty acids shown to be oxidized by *S. marcescens* produces organisms which have no significant lag period in the oxidation of the other acids. If certain acids were oxidized by adaptive enzymes and others by constitutive enzymes, one would not expect this reciprocal adaptation to occur, and it is probable that the enzymes involved in the oxidation of these acids are either all adaptive or all constitutive. It is known that dried cells prepared from caprate grown cells will oxidize all the fatty acids oxidized by living cells, whereas dried cells prepared from glucose grown cells do not oxidize any of the fatty acids (Silliker, 1951). This again suggests that the formation of the fatty acid oxidation system in *S. marcescens* is an adaptive process, but it leaves unexplained the anomalous results of apparent adaptation to C_{10}, C_{12}, and C_{14} acid oxidation in the presence of 2,4-dinitrophenol.

SUMMARY

2,4-Dinitrophenol directly blocks the oxidation of the 2,6, and 7 carbon fatty acids by both adapted and unadapted cells of *Serratia marcescens*. It prevents adaptation to the oxidation of the 8, 9, 11, and 13 carbon acids but not adaptation to oxidation of the 10, 12, and 14 carbon acids. Where oxidation proceeds in the presence of 2,4-dinitrophenol, oxidative assimilation is partially or completely blocked. Both in the presence and absence of 2,4-dinitrophenol the portion of the fatty acids not oxidatively assimilated apparently is oxidized to completion without the accumulation of unoxidized fragments. Similar results were obtained with sodium azide.

The evidence presented shows that acetic, caproic, and caprylic acids are not direct intermediates in the oxidation of the C_{10}, C_{12}, and C_{14} fatty acids by *S. marcescens*, and acetic and heptylic are not intermediates in C_9, C_{11}, and C_{13} oxidation. It thus appears that beta oxidation of fatty acids is not the mechanism utilized by *S. marcescens*.

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