STUDIES ON THE AEROBIC OXIDATION OF FATTY ACIDS BY BACTERIA

II. Application of the Technique of Simultaneous Adaptation to the Study of the Mechanism of Fatty Acid Oxidation in Serratia Marcescens[1]

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In the preceding paper (Silliker and Rittenberg, 1951*a*) it was reported that *Serratia marcescens* (Alphin) oxidizes a variety of straight-chain saturated fatty acids. Cells harvested from a glucose mineral salts medium show an appreciable lag in oxygen uptake; on the other hand, cells grown on a medium containing a particular fatty acid as the sole source of carbon show no significant lag period in the oxidation of that acid. These findings suggested that the oxidation of fatty acids by this particular strain of *Serratia marcescens* is catalyzed by adaptive enzymes and hence that this organism might be a valuable tool for a study of the mechanism of fatty acid oxidation if one uses the technique of simultaneous adaptation (Stanier, 1947).

Since little is known of the mechanisms of aerobic attack on fatty acids by bacteria, it might be assumed, for the purposes of selecting possible intermediates, that a mechanism analogous to one of those thought to occur in animal tissue is operative, i.e., *beta*, multiple alternate, or *omega* oxidation. If classical *beta* oxidation were to occur, for instance, then adaptation of cells to the oxidation of a ten-carbon acid should result in simultaneous adaptation to its eight- and six-carbon homologues; similarly, adaptation to the oxidation of a nine-carbon acid should simultaneously adapt organisms to the seven- and five-carbon acids. If, on the other hand, either *omega* or multiple alternate oxidation were to occur, then adaptation of cells to any particular fatty acid should not necessarily adapt cells to lower homologues, since lower fatty acids would not occur as intermediates in oxidation.

EXPERIMENTAL RESULTS

Oxidative patterns in relation to growth on capric acid medium. Serratia marcescens was grown on medium A (Silliker and Rittenberg, 1951*a*) with 0.01 M capric acid as the only carbon source. Cells were harvested after 40 hours of growth at 37 C by washing down the agar slants with M/20 phosphate buffer. The cells were washed twice by centrifugation and resuspension in fresh buffer, and the final

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suspension was adjusted to a turbidity of 300 on the Klett-Summerson apparatus, using the 540-mμ filter. The caprate-grown cells were then tested for the oxidation

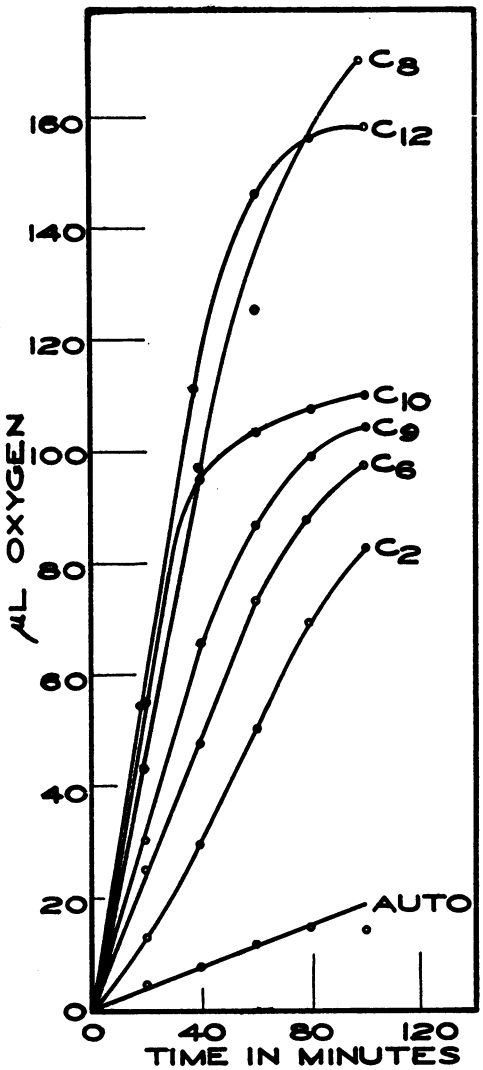

Figure 1. Oxidation of various fatty acids by caprate-grown cells of *S. marcescens*. M/20 phosphate buffer, pH 7.0, 30 C, air atmosphere with 0.1 ml 10 per cent KOH in center well. Substrate quantities: 4 μM acetate, 1 μM caproate and caprylate, 0.6 μM pelargonate, caprate, and laurate. Propionate, butyrate, and valerate were not oxidized.

of various fatty acids, using conventional manometric techniques (Umbreit *et al.*, 1949).

Figure 1 shows that cells harvested from capric acid medium consume oxygen immediately and at the maximum rate when exposed to capric acid, the lag period noted when glucose-grown cells oxidize the same compound being absent

(figure 2 of the preceding paper). Likewise, caprate-grown cells show no lag period in the oxidation of caprylic (C_8), caproic (C_6), and acetic (C_2) acids. If the oxidation of these substances is catalyzed by adaptive enzymes, then the simultaneous adaptation of caprate-grown cells to the oxidation of caprylate, caproate, and acetate would indicate that these substances are intermediates in the oxidation of the ten-carbon acid. On the basis of these findings alone, one would be led to the conclusion that *beta* oxidation occurs. Certain other aspects of the experimental results indicate that this conclusion would be premature: (1) Butyrate is not oxidized by either glucose or caprate-grown cells; yet this acid would be an intermediate if *beta* oxidation were to occur. (2) Caprate-grown cells are simultaneously adapted to the oxidation of pelargonate, a nine-carbon acid that could not be an intermediate in *beta* oxidation, and to the oxidation of laurate, a twelve-carbon acid that could not possibly be a direct intermediate under any circumstances.

Oxidative patterns in relation to specific adaptation to various fatty acids. Although adaptation of *Serratia marcescens* cells to capric acid resulted in simultaneous adaptation to feasible intermediates in caprate oxidation, the cells also became adapted to compounds that could not possibly be considered intermediates. In order to investigate further the effect of specific adaptation on oxidative patterns, cultures of *Serratia marcescens* (Alphin) were adapted in turn to each of the oxidizable fatty acids. The specifically adapted cells were then tested for the oxidation of other fatty acids.

It was found that adaptation could be accomplished either by growth on a medium containing a fatty acid as the sole source of carbon or by allowing buffer suspensions of glucose-grown cells to oxidize a small amount of fatty acid in the Warburg flask (exposure). There was no essential difference between cells adapted by growth and those adapted by exposure, although cells adapted by exposure had a somewhat lower rate of oxidation than organisms grown on the compound in question. Since growth on fatty acid medium was in some cases slow and irregular and in others completely negative (acetate and heptylate), most of the experiments were conducted with exposed cells. Under these conditions, 16- to 18-hour glucose-grown cells could be used. Cells were washed twice with phosphate buffer and then suspended in buffer containing a small amount of fatty acid (1.0 μM per ml for acetate oxidation, 0.6 μM per ml for all other acids). The cells were allowed to oxidize the added acid to completion; when the autorespiratory level was reached, test substrate was poured from the side arm into the main flask containing the adapted cells.

Figure 2 shows the oxidation of capric acid by cells specifically adapted to various other fatty acids. It will be noted that adaptation of cells to any one of the oxidizable acids has a pronounced effect on the pattern of caprate oxidation. In most instances the lag period is eliminated, oxygen uptake beginning immediately and at the maximum rate. Heptylate-exposed cells show a definite lag period (16 minutes) before caprate is oxidized at the maximum rate, but it is evident that these cells reach the maximum rate of oxidation much more rapidly than do unadapted cells.

Table 1 shows that adaptation of *Serratia marcescens* cells to any one of the

oxidizable acids has a profound effect on the pattern of oxidation of all the other acids. The procedure eliminates or reduces the length of the lag period that is observed when glucose-grown (unadapted) cells oxidize the same compounds. A considerable variation exists in the degree to which adaptation to a particular acid shortens the lag period in the oxidation of other acids. In many cases, oxygen is consumed at the maximum rate from the start, indicating that complete adaptation has occurred. In other instances, the length of the lag period is inter-

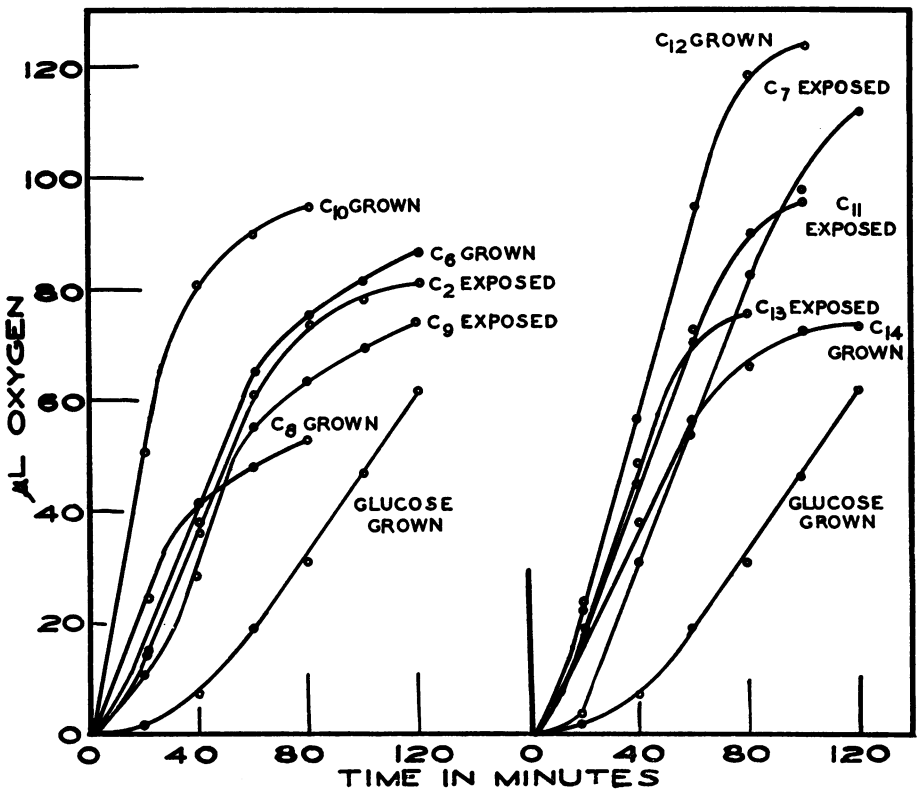

Figure 2. Oxidation of caprate by *S. marcescens* grown on or exposed to other fatty acids. M/20 phosphate buffer, pH 7.0, 30 C, air atmosphere with 0.1 ml 10 per cent KOH in center well, 0.6 μM capric acid per flask.

mediate between that for unadapted cells and that for completely adapted cells. There appears to be no definite pattern in complete and partial adaptation. At present the significance of lag periods of both short and intermediate length is in doubt. There is in these data, however, definite evidence that as a result of exposure to or growth on any of the oxidizable acids there occurs a general stimulation of fatty acid oxidation in *Serratia marcescens*.

Studies on the oxidation of caprate intermediates. All proposed mechanisms of fatty acid oxidation involve the formation of *beta*-keto acids at some stage in catabolism. Although unequivocal proof as to the mechanism of their formation

is lacking, the following sequence of reactions appears most likely in animal tissue (Breusch, 1948) and in molds (Foster, 1949):

$$R—CH_2—CH_2—COOH \xrightarrow{-2H} R—CH=CH—COOH$$
$$\xrightarrow{+H_2O} R—CHOH—CH_2—COOH \xrightarrow{-2H} R—CO—CH_2—COOH.$$

For the purpose of studying the primary reactions in fatty acid catabolism by *Serratia marcescens*, the *alpha-beta* unsaturated,[3] *beta*-hydroxy,[4] and *beta*-keto[5] derivatives of capric acid were prepared. The oxidation of these substrates was studied in a manner similar to that used in the study of the oxidation of normal saturated acids.

TABLE 1

Lag periods in the oxidation of fatty acids in relation to specific adaptation of Serratia marcescens (Alphin) cells to various acids

OXIDATION OF	LAG PERIOD* IN MINUTES AFTER SPECIFIC ADAPTATION OF SERRATIA CELLS TO										
	Control†	C_2	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}
Acetic	20	5	2	8	6	0	8	7	6	8	14
Caproic	28	5	7	18	2	18	2	xx	5	10	6
Heptylic	19	8	9	7	0	5	9	0	8	xx	14
Caprylic	39	0	4	11	1	12	3	7	6	4	5
Pelargonic	29	0	9	xx	0	1	3	xx	4	xx	4
Capric	27	8	8	16	0	16	4	7	7	5	0
Undecylic	32	3	xx	xx	xx	xx	0	0	xx	9	xx
Lauric	40	20	8	xx	0	28	6	xx	0	xx	xx
Tridecylic	14	7	0	9	6	9	0	8	0	1	10
Myristic	34	9	18	17	0	20	3	6	0	7	17

Air atmosphere with 10 per cent KOH to absorb CO_2; flask concentration of acetic acid 0.001 M; other acids, 0.0003 M; M/20 phosphate buffer, pH 7.0; temperature, 30 C.

xx—no data available.

* Lag periods estimated by extension of steepest part of the curve to time axis. Most values represent averages from several experiments.

† Glucose-grown cells; not exposed to any acid.

Figures 3, 4, and 5 show the oxidation of caprate derivatives by *Serratia marcescens* cells of different histories. It can be seen that glucose-grown cells oxidize each of the three substrates only after a significant lag period. Cells harvested from capric acid medium show no lag period in the oxidation of *beta*-ketocapric acid; the lag periods in the oxidation of the unsaturated and hydroxy derivatives were almost eliminated. If the lag periods represent a time during which adaptive enzymes are being formed, then it appears that *beta*-ketocapric acid is an intermediate in caprate oxidation. Since the particular batch of caprate-grown cells used in this experiment showed a 4-minute lag period in the oxidation of caprate, it is reasonable to consider the short lag periods observed in the oxidation of the

[3] Prepared according to the method of Thaler and Geist (1939).

[4] Prepared according to the method of Stenhagen (1945).

[5] Prepared according to the method of Tulus (1944).

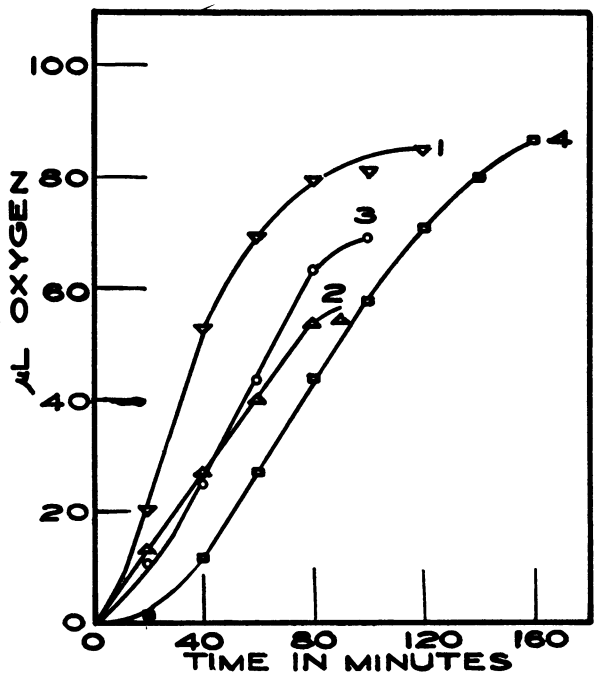

Figure 3. Oxidation of *alpha-beta* unsaturated capric acid by *S. marcescens*: (1) caprate-grown; (2) glucose-grown, *beta*-hydroxycapric-acid-exposed; (3) glucose-grown, *beta*-ketocapric-acid-exposed; (4) glucose-grown. M/20 phosphate buffer, pH 7.0, 30 C, air atmosphere with 0.1 ml 10 per cent KOH in center well, 0.6 μM *alpha-beta* unsaturated capric acid per flask.

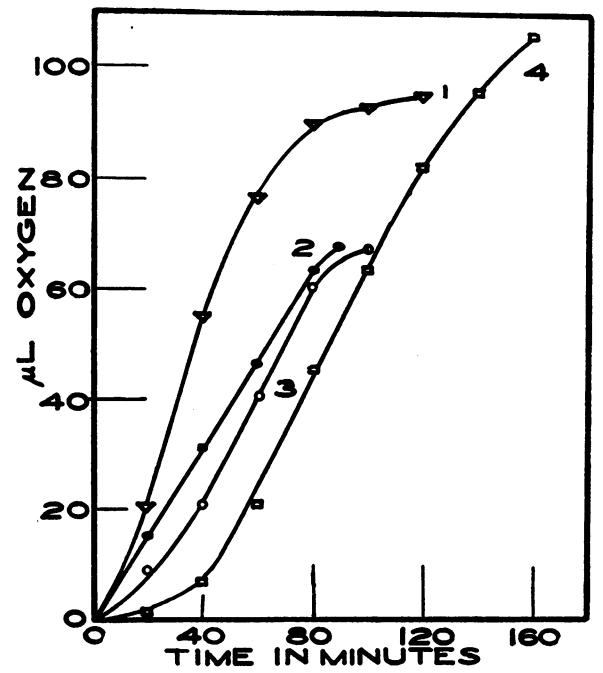

Figure 4. Oxidation of *beta*-hydroxycapric acid by *S. marcescens*: (1) caprate-grown; (2) glucose-grown, *alpha-beta*-unsaturated-capric-acid-exposed; (3) glucose-grown, *beta*-ketocapric-acid-exposed; (4) glucose-grown. M/20 phosphate buffer, pH 7.0, 30 C, air atmosphere with 0.1 ml 10 per cent KOH in center well, 0.6 μM *beta*-hydroxycapric acid per flask.

unsaturated and hydroxy derivatives as being due to technical difficulties in the conduct of the experiment. One can conclude, therefore, that growth on capric acid medium adapts cells to the unsaturated and hydroxy derivatives and that these also are intermediates in caprate oxidation.

Figures 3, 4, and 5 also show that exposure of glucose-grown cells to any of the three derivatives eliminated or almost eliminated the lag periods in the oxidation of the other two compounds. It will be noted that the rate of oxidation by the exposed cells was somewhat lower in each case than the corresponding rate ob-

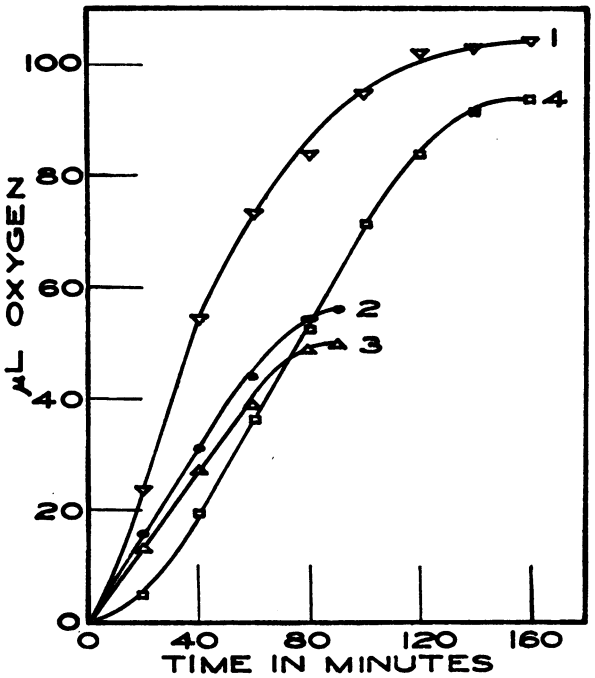

Figure 5. Oxidation of *beta*-ketocapric acid by *S. marcescens*: (1) caprate-grown; (2) glucose-grown, *alpha-beta*-unsaturated-capric-acid-exposed; (3) glucose-grown, *beta*-hydroxycapric-acid-exposed; (4) glucose-grown. M/20 phosphate buffer, pH 7.0, 30 C, air atmosphere with 0.1 ml 10 per cent KOH in center well, 0.6 μM *beta*-hydroxycapric acid per flask.

served with caprate-grown cells. This higher activity by caprate-grown cells can probably be attributed to a quantitative difference between enzyme content in exposed cells and caprate-grown cells. The data reveal little with respect to the sequence of reactions, but chemical logic would dictate that, if all three compounds are intermediates, then the unsaturated acid is formed directly from caprate, with the formation of the hydroxy and keto acids following in order.

Cells adapted to the three caprate derivatives were tested for the oxidation of caprate, caprylate, and undecylate. Table 2 shows that cells adapted to any one of the three caprate derivatives were also adapted to the oxidation of the C_8, C_{10},

and C_{11} acids. As in other studies, there were instances in which the lag periods were not completely eliminated, but the great difference between the lag periods for exposed and unexposed cells allows the conclusion that exposure to any one of the three compounds adapts cells to the oxidation of the three normal fatty acids tested.

TABLE 2

Lag periods in the oxidation of capric, undecylic, and caprylic acids in relation to adaptation to capric acid derivatives

OXIDATION OF	LAG PERIOD IN MINUTES AFTER SPECIFIC ADAPTATION TO			
	Alpha-beta unsaturated capric acid	*Beta*-hydroxycapric acid	*Beta*-ketocapric acid	Unadapted
Caprylic	5	3	6	25
Capric	0	0	7	30
Undecylic	2	6	4	32

Specific adaptation was accomplished by exposing *S. marcescens* (Alphin) cells to 0.6 μM of derivative per ml of cell suspension. Unadapted cells were unexposed. All cells were glucose-grown.

Flask concentrations of fatty acids were 0.0003 M. Experiments were conducted in air atmosphere with 10 per cent KOH in center well. Temperature 30 C.

Lag periods were calculated by extension of the steepest part of the curve to the time axis.

DISCUSSION

The occurrence of a lag period in oxygen uptake or in carbon dioxide production by microorganisms when exposed to a particular substrate has been the major criterion for concluding that adaptive enzymes are involved in the oxidation of the compound in question. In the present study, the lag periods in oxygen uptake that were observed when glucose-grown cells attacked fatty acids and the elimination of these lag periods as a result of growth on fatty acids were considered good presumptive evidence that the oxidation of these compounds by *Serratia marcescens* is catalyzed by adaptive enzymes. However, the peculiar results obtained in these investigations demand the consideration of other possible explanations for the lag periods, even though the initial hypothesis of adaptive enzyme involvement still seems most probable.

Permeability as a possible factor in the lag periods. Breusch (1948) has pointed out that insolubility and slow diffusibility are factors that make it difficult to bring fatty acids in contact with intracellular enzymes. It is conceivable that the glucose-grown cells are permeated by fatty acids at a slow rate and that this characteristic is reflected in an initial lag in oxygen uptake. The major difference between adapted and unadapted cells would then be a difference in permeability and not in enzymatic constitution, i.e., the enzymes catalyzing fatty acid oxidation are constitutive. As a result of growth on or exposure to a fatty acid, the permeability characteristics of the cell would have to change with respect to all other fatty acids known to be metabolized by these organisms, since this process

either partially or completely eliminates the lag period in the oxidation of fatty acids in general. Further, if the difference between adapted and unadapted cells is one of permeability, then one should be able to demonstrate the fatty acid oxidation system with equal facility in glucose-grown and fatty-acid-grown cells under conditions in which enzyme synthesis is blocked as in the presence of cell poisons or with cell-free preparations. Further, dry cell preparations from glucose-grown cells should oxidize fatty acids without a lag period, since under these conditions slow permeability should not be a factor. Experiments dealing with these points and those raised in the next section are under investigation and will be reported later.

The possible accumulation of a key substrate. The possibility suggests itself that the lag periods observed with glucose-grown cells represent a time during which some key compound or compounds necessary for fatty acid oxidation are accumulating in the cell. Such a substance might arise either through the oxidation of a small amount of fatty acid during the lag period or from the breakdown of storage material. Of particular interest in this respect is the position of the tricarboxylic acid cycle. It has been shown that this cycle is involved at two distinct points in the catabolism of fatty acids by animal tissue: (1) Grafflin and Green (1948) and Knox *et al.* (1948) have demonstrated that the oxidation of a small amount of tricarboxylic acid cycle compound is an obligatory "sparking reaction" in fatty acid oxidation by the cyclophorase system. (2) When fatty acids are oxidized to completion, the terminal oxidation involves a coupling reaction (probably with oxalacetate), and the condensation product undergoes oxidation to carbon dioxide and water through the tricarboxylic acid cycle (Breusch, 1948). It is possible that similar reactions occur in *S. marcescens*, in which case the lag period might represent a time during which a small amount of tricarboxylic acid cycle compound is being oxidized and that this reaction "sparks" fatty acid oxidation. Alternatively, the absence of sufficient tricarboxylic acid cycle substrate could be limiting the rate of fatty acid oxidation at a later point in the catabolic process, i.e., at the condensation stage. In the first instance, one would expect that cells harvested from fatty acid medium would already be "sparked" with respect to fatty acid oxidation. On the other hand, if low concentrations of tricarboxylic acid cycle compounds limit the rate of oxidation at a later stage, then cells harvested from fatty acid medium should have a larger store of these compounds than do glucose-grown cells. The involvement of the tricarboxylic acid cycle in either manner would imply that true enzymatic adaptation is not involved in fatty acid oxidation by *S. marcescens*.

The possibility of a common enzyme system. A third, and at the moment the most probable, explanation for the lag periods appears to be that during this phase there is a synthesis of enzymes directly concerned with attack on the fatty acid molecule. If such be the case, then the theory behind the concept of simultaneous adaptation applies. Since growth on or exposure to any oxidizable fatty acid simultaneously adapts to the oxidation of all other oxidizable fatty acids, one must conclude that a single enzyme system initiates attack on all fatty acids. This is confirmed by the fact that exposure of glucose-grown cells to

alpha-beta unsaturated capric acid and to the other caprate derivatives results in adaptation not only to the oxidation of caprylic acid, a possible intermediate, but also to undecylic acid, an 11-carbon acid that could not possibly be an intermediate in the oxidation of the 10-carbon acid.

The postulate of a single enzyme system in which the individual enzymes function in a repeating sequence will fit into either the *beta* or the multiple alternate oxidation mechanism. The initial steps are the same in both schemes. They involve (1) the conversion of saturated acid to *alpha-beta* unsaturated acid by the removal of two hydrogen atoms, (2) the conversion of unsaturated acid to *beta*-hydroxy acid by the addition of water at the *alpha-beta* position, and (3) the conversion of hydroxy acid to keto acid by the removal of two more hydrogen atoms.

At this point the keto acid would undergo cleavage to give a two-carbon fragment and a compound with two less carbon atoms. The lower compound is oxidized through the same pathway as the higher homologue; enzymes catalyzing reactions 1, 2, and 3 again come into play in the same sequence. Although this mechanism implies classical *beta* oxidation with normal acids being degraded at the rate of two carbon atoms at a time to the next lower acid, data to be presented in a future communication indicate that this is not the case. Studies with cell poisons (2,4-dinitrophenol and sodium azide) have shown that caprylate, caproate, and acetate are not direct intermediates in the oxidation of caprate. Likewise heptylate is not a direct intermediate in the oxidation of pelargonate (Silliker and Rittenberg, 1951*b*). This does not rule out the possibility that compounds readily formed from the normal C_8, C_6, C_2, and C_7 acids are direct intermediates in the oxidation of the C_{10} and C_9 acids. If such proves to be the case, then the essential features of classical *beta* oxidation are retained, but in *S. marcescens* the chemical nature of the intermediate compounds formed in the process must be different from that predicted on the basis of Knoop's theory.

Alternatively the keto acid formed as a result of reactions 1, 2, and 3 does not cleave, but a second oxidation occurs at the *delta* carbon atom. As a result, after a *gamma-delta* unsaturation and *delta*-hydroxy acid formation a second keto group is formed; subsequently (or simultaneously) similar reactions occur at alternate carbon atoms on the fatty acid with the eventual formation of a polyketo acid. The same enzymes catalyzing reactions 1, 2, and 3 catalyze the analogous reactions involved in the formation of the *delta* and subsequent keto groups. The experimental data do not, as yet, allow a choice between the two mechanisms suggested; no alternative pathway is evident. Either mechanism explains the simultaneous adaptation to all oxidizable fatty acids and intermediates as a result of adaptation to any compound anywhere in the reaction sequence.

Accepting the hypothesis of a single enzyme system being involved in the primary attack on fatty acids, it is nevertheless difficult to visualize acetic acid being metabolized in the same manner. The details of acetate metabolism in animal tissues remain to be discovered, but there is general agreement that the tricarboxylic acid cycle is involved (Green, 1948). Saz and Krampitz (1950) have

given evidence for a similar pathway in *Micrococcus lysodeikticus*. Karlsson and Barker (1948) and Ajl (1950) have presented evidence against the occurrence of the tricarboxylic acid cycle in *Azotobacter agilis* and *Escherichia coli*. Ajl has suggested an alternative pathway involving condensation of two acetate molecules with the production of succinic acid. A similar formation of succinate from acetate has been suggested by Nord and Vitucci (1947), Randles and Birkland (1947), Slade and Werkman (1943), and Barron *et al.* (1950). There thus appear to be at least two possible pathways of acetate oxidation in microorganisms; the enzymes catalyzing the reactions involved must be quite unrelated to those active in the initial steps of higher fatty acid oxidation. Yet in the present study it was found that *S. marcescens* cells that had been exposed to acetate were completely or partially adapted to the oxidation of higher fatty acids.

Stanier *et al.* (1950) have recently considered the possibility of two or more distinct reaction chains, one of which is reversible, merging in a common intermediate. They point out that adaptation to any compound on one chain would cause complete adaptation to the common intermediate and consequently the possibility of back adaptation to the freely reversible chain of reactions would exist. In the light of this reasoning, it seems possible that the reaction chains in the oxidation of higher fatty acids merge at a common point, perhaps acetate. If the reactions are reversible, as suggested by the work of Rittenberg and Bloch (1945) on fatty acid synthesis from acetate, then adaptation to the common intermediate acetate might be expected to cause back adaptation to the oxidation of higher acids.

The question also arises as to why propionic, butyric, and valeric acids are not oxidized by *S. marcescens* cells that are capable of oxidizing higher fatty acids. Work with dry cells prepared according to the method of Sleeper *et al.* (1950) has shown that failure to demonstrate oxidation of the three- and five-carbon acids is due to impermeability of the living cells to these acids rather than to a lack of appropriate enzymes for their oxidation (Silliker and Rittenberg, (1951*b*). The failure of either living or dried cells to oxidize butyric acid remains unexplained.

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SUMMARY

Glucose-grown cells of *Serratia marcescens* (Alphin) oxidized all straight-chain saturated fatty acids from acetic through myristic with an appreciable lag period with the exceptions of propionic, butyric, and valeric, which were not oxidized at all. Cells grown on a medium containing one of the fatty acids as the sole source of carbon or cells allowed to oxidize a small amount of one of the fatty acids showed no lag period or a greatly reduced lag period in the oxidation of the homologous acid as well as in the oxidation of all the other fatty acids.

Alpha-beta unsaturated, *beta*-hydroxy, and *beta*-ketocapric acid were also oxidized after a considerable lag by glucose-grown *S. marcescens* cells. Growth on caprate eliminated the lag period in the oxidation of the caprate derivatives. Exposure to any of the caprate derivatives eliminated or greatly reduced the lag periods in the oxidation of the other caprate derivatives and caprate, caprylate, and undecylate.

Several possible explanations for the observed lag periods and their elimination are discussed. It is postulated that a single enzyme system, in which the individual enzymes function in a repeating sequence, catalyze the degradation of all the fatty acids oxidized by *S. marcescens* (acetic acid excluded). The data are consistent with either multiple alternate oxidation or a somewhat modified form of *beta* oxidation; no choice between the two mechanisms is possible as yet.

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