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Control of Fatty Acid Metabolism

I. Induction of the Enzymes of Fatty Acid Oxidation in *Escherichia coli*

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Escherichia coli grows on long-chain fatty acids after a distinct lag phase. Cells, preadapted to palmitate, grow immediately on fatty acids, indicating that fatty acid oxidation in this bacterium is an inducible system. This hypothesis is supported by the fact that cells grown on palmitate oxidize fatty acids at rates 7 times faster than cells grown on amino acids and 60 times faster than cells grown on a combined medium of glucose and amino acids. The inhibitory effect of glucose may be explained in terms of catabolite repression. The activities of the five key enzymes of β-oxidation [palmityl-coenzyme A (CoA) synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydrase, β-hydroxyacyl-CoA dehydrogenase, and thiolase] all vary coordinately over a wide range of activity, indicating that they are all under unit control. The ability of a fatty acid to induce the enzymes of β-oxidation and support-growth is a function of its chain length. Fatty acids of carbon chain lengths of C_{14} and longer induce the enzymes of fatty acid oxidation and readily support growth, whereas decanoate and laurate do not induce the enzymes of fatty acid oxidation and only support limited growth of palmitate-induced cells. Two mutants, D-1 and D-3, which grow on decanoate and laurate were isolated and were found to contain constitutive levels of the β-oxidation enzymes. Short-chain fatty acids ($<C_8$) do not support growth of either the parent strain or the mutants D-1 and D-3. Evidence is also presented to show that decanoate is actively transported by the parent strain and by the mutants.

Our knowledge of the mechanism of fatty acid oxidation has been gained largely from studies on animal tissue (4, 9). The reactions involved can be delineated as follows:

$$RCH_2CH_2COOH + CoASH + ATP \rightleftharpoons RCH_2CH_2COSCoA + H_2O + PP_i + AMP \quad (1)$$

$$RCH_2CH_2COSCoA + FAD\text{-}E \rightarrow RCH = CHCOSCoA + FADH_2\text{-}E \quad (2)$$

$$RCH=CHCOSCoA + H_2O \rightleftharpoons L(+)RCHOHCH_2COSCoA \quad (3)$$

$$L(+)RCHOHCH_2COSCoA + NAD \rightleftharpoons RCOCH_2COSCoA + NADPH + H^+ \quad (4)$$

$$RCOCH_2COSCoA + CoASH \rightleftharpoons RCOSCoA + CH_3COSCoA \quad (5)$$

Fatty acid is first activated (reaction 1) with coenzyme A (CoA) to form acyl-CoA by an adenosine triphosphate (ATP)-dependent reaction catalyzed by the acyl-CoA synthetase [acid: CoA ligase, adenosine monophosphate (AMP), EC 6.2.1.3]. The acyl-CoA is then oxidized (reaction 2) to 2-enoyl-CoA by the action of the acyl-CoA dehydrogenase (acyl-CoA: cytochrome *c* oxidoreductase, EC 1.3.2.2). The enzyme enoyl-CoA hydrase (L-3-hydroxyacyl-CoA hydro-lyase, EC 4.2.1.17) hydrates (reaction 3) the 2-enoyl-CoA to β-hydroxyacyl-CoA and this is then oxidized (reaction 4) to β-keto acyl-CoA by the enzyme β-hydroxyacyl-CoA dehydrogenase [L-3-hydroxyacyl-CoA: nicotinamide adenine dinucleotide (NAD) oxidoreductase, EC 1.1.1.35]. Finally, the β-keto-acyl-CoA is cleaved (reaction 5) by the enzyme thiolase (acetyl-CoA: acetyl-CoA C acetyl transferase, EC 2.3.1.9) to acetyl-CoA and an acyl-CoA with two carbons fewer than the original acyl-CoA. This acyl-CoA is then recycled through the same sequence of reactions until eventually it is completely degraded to acetyl-CoA.

In spite of the fact that studies with bacteria have, in recent years, made considerable contributions to the understanding of the mechanism

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of fatty acid synthesis (11, 20), there have been few studies of bacterial fatty acid oxidation (12). Recently, however, Overath et al. (13) reported the induction of some of the enzymes of β-oxidation and isolated mutants which were unable to oxidize fatty acids.

In view of the great contribution that bacterial genetics has made to the clarification of metabolic control mechanisms and because of our continued interest in fatty acid metabolism, a study of fatty acid oxidation and its relationship to fatty acid synthesis has been undertaken to provide insight into the regulation of fatty acid metabolism. *Escherichia coli* was chosen because of our detailed knowledge of its fatty acid synthesis and the wealth of available genetic information concerning this organism. The present report describes the utilization of various fatty acids by *E. coli* and the coordinate induction of the five enzymes of β-oxidation by long-chain fatty acids. It also describes the isolation of mutants with constitutive levels of the β-oxidation enzymes.

MATERIALS AND METHODS

Chemicals. Fatty acids were products of the Hormel Institute, Austin, Minn. ^{14}C-fatty acids were purchased from New England Nuclear Corp., Boston, Mass. Pig heart β-hydroxyacyl-CoA dehydrogenase was purchased from Calbiochem, Los Angeles, Calif. All other reagents were obtained commercially and were used without further purification. Acetoacetyl-CoA and crotonyl-CoA were prepared by reacting reduced CoA at *p*H 8.0 with diketene and crotonic anhydride, respectively. Palmityl-CoA was purchased from P-L Biochemicals.

Organism. The strain of *E. coli* used throughout this work was SR 258, a derivative of *E. coli* K-12, carrying the following markers: F$^-$, (*thr*$^-$, *leu*$^-$, *lac*$^-$, *malA*$^-$, *tsx*$^-$, *thi*$^-$).

Media and conditions of cultivation. Cells were grown either in Nutrient Broth (Difco) or in a synthetic medium containing, in addition to a carbon source: Triton X-100, 10 ml; $MgSO_4$, 0.1 g; K_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 1 g; thiamine, 5 mg; threonine, 40 mg; and leucine, 40 mg; in a volume of 1 liter. Carbon sources were added in the following quantities: fatty acid, 1 g; glucose, 5 g; glycerol, 2 g; and synthetic amino acid mixture (15), 2 g. When a mixture of carbon sources was used, each was included in the amounts shown above unless otherwise indicated. Cells were grown at 37 C in a New Brunswick gyratory shaker, model G25.

Nephalo culture flasks (Bellco Glass Inc., Vineland, N.J.) containing 25 to 30 ml of medium were used for studying the growth characteristics on the various carbon sources. Media were inoculated to an initial turbidity of 10 to 20 Klett units with saline suspensions of washed cells grown to mid-exponential phase in a medium containing either the amino acid mixture or palmitate. Growth was monitored as turbidity, by use of a Klett-Summerson colorimeter equipped with a blue filter. For preparations of the cell-free extracts, cells were grown in 1 liter of medium to late exponential phase. The cells were harvested by centrifugation at 35 C and were washed twice with 2 liters of warm (35 C) 0.05 M potassium phosphate buffer (pH 7.4) containing 1% (v/v) Triton X-100.

Preparation of cell-free extracts. Washed cells, prepared from 1-liter cultures, were resuspended either in 0.05 M potassium phosphate (pH 7.4) containing 0.01 M mercaptoethanol or in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 7.0). Extracts prepared in the first buffer were used for the assay of the overall oxidative activity, whereas those prepared in Tris-chloride were used for the assay of the individual enzymes. Sufficient volume of buffer was added to give a concentration of about 50 mg (dry weight) of cells/ml. The cells were disrupted with a Branson Sonifier for 1 min. The sonic treatment was applied for 15-sec intervals with the use of intermittent cooling to maintain the temperature of the cells below 4 C. The resulting suspension was centrifuged at 10,000 × *g* for 30 min. The supernatant liquid was decanted and used for the various enzymatic assays. The protein concentration of the supernatant liquid was determined by the biuret method (3).

Uptake of decanoate by resting cells. Washed cells were resuspended in a sufficient volume of basic medium to give a concentration of 10 mg (dry weight) of cells/ml. The basic medium consisted of: K_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 1 g; Triton X-100, 10 ml; and water to a final volume of 1 liter. Portions of the suspension of cells (25 μliters) were diluted to 0.5 ml with basic medium containing 50 nmoles of decanoate-*1*-^{14}*C* (300,000 counts/min). The reaction mixtures were incubated at 37 C for various time intervals. Each reaction was terminated by rapid filtration of the cells through an ice-cold membrane filter (HA, 0.45 μm; Millipore Corp., Bedford, Mass.), and excess decanoate-*1*-^{14}*C* was removed by washing the cells three times with 1 ml of ice-cold basic medium. At zero time, an equivalent portion of cells was added to a reaction mixture at 0 C, immediately filtered, and washed as described above. The membrane filters were dried under an infrared lamp, added to a vial containing scintillation mixture, and counted.

In some experiments, cells were incubated for 20 sec as described above and 0.5 ml of 0.01 M nonlabeled decanoate in basic medium was added. The reactions were incubated for a further 10 or 20 sec, and the cells were filtered and washed.

Assay of fatty acid oxidation in resting cells. Washed cells, prepared from 250 ml cultures, were resuspended in 0.05 M potassium phosphate (*p*H 7.4) containing 0.01 M mercaptoethanol, to a final concentration of about 5 mg (dry weight) of cells/ml. Fatty acid oxidation was assayed by determining the amount of $^{14}CO_2$ formed from fatty acid-*1*-^{14}*C* by the method of Bressler and Friedberg (1). Each preparation of cells was assayed at three different levels of cells (0.1, 0.2, and 0.3 mg, dry weight), a range over which the activity was linear. Each reaction mixture contained 1 ml of freshly oxygenated Krebs-Ringer phosphate (*p*H 7.4) (2), 100 nmoles of fatty acid-*1*-^{14}*C* (50,000 counts/min), the indicated amounts of cells, and

water to a final volume of 2.0 ml. The reaction mixtures, in 25-ml Erlenmeyer flasks with glass center wells, were maintained at 0 C, and a polyethylene well (Kontes Glass Co., Vineland, N.J.) was inserted within the center well so that there was minimal contact between it and the reaction mixture. Each flask was sealed with a rubber serum stopper, placed in a Eberbach waterbath shaker, and incubated at 25 C for 1 hr. The reaction was terminated by the injection of 0.15 ml of 6 N H_2SO_4 through the rubber stopper. Hyamine (0.2 ml) was then injected into the polyethylene insert, and the reaction mixture was shaken for an additional 1 hr. The hyamine-containing insert was carefully removed, wiped, and added to a counting vial containing 10 ml of scintillation fluid. Radioactivity was measured in a Packard liquid scintillation counter. Internal quenching by the plastic insert was negligible. The mean specific activity was calculated from the three independent assays and was expressed as nanomoles of palmitate oxidized per minute per milligram (dry weight) of cells.

Assay of fatty acid oxidation in cell-free extracts. The assay procedure was identical to that described for the resting cells. The reaction mixture contained: 1.0 ml of freshly oxygenated Krebs-Ringer phosphate, *p*H 7.4 (2); palmitate-*1*-^{14}C, 20 nmoles (80,000 counts/min); CoA, 1 μmole; NAD, 1 μmole; ATP, 1 μmole; succinate, 10 nmoles; supernatant protein, 1 to 5 mg; and water to a final total volume of 2.0 ml.

Little oxidation was observed when less than 1 mg of protein was used. However, significant oxidation rates were observed with more than 1 mg of protein, and the rates were linear up to 5 mg. The addition of boiled extract to the incubation mixtures had no effect on the oxidation rates at any of the protein concentrations. Routinely, assays were performed at three different levels of protein. The specific activity was calculated as the mean of the three determinations and expressed as picomoles of palmitate oxidized per minute per milligram of protein.

Assay of the enzymes of β-oxidation. The activity of each enzyme was assayed at two concentrations of protein within the indicated range. The rate of reaction of each enzyme was proportional to the amount of added protein. Specific activity was calculated as the mean of the two values and expressed as nanomoles of substrate utilized per minute per milligram of protein.

Palmityl-CoA synthetase. The assay employed was a modification of the method of Kornberg and Pricer (8). Each assay mixture contained: ATP, 8 μmoles, CoA, 0.56 μmoles; $MgCl_2$, 4.0 μmoles; salt-free hydroxylamine (*p*H 7.4), 250 μmoles; palmitate-*1*-^{14}C, 1 μmole (150,000 counts/min); protein, 0.25 to 1.0 mg; and water to a final volume of 1 ml. Reaction mixtures were incubated in centrifuge tubes for 1 hr at 37 C. Ice-cold 7% perchloric acid (0.7 ml) was added, and the tubes were stored for 15 min at 0 C. The precipitate was removed by centrifugation and washed once with 1.0 ml of 3.5% perchloric acid. The washed precipitate was suspended in 1.0 ml of 0.1% Hill's solution (6) in methanol, and the suspension was incubated at 37 C for 30 min. The mixture was centrifuged, and the supernatant solution was extracted three times with an equal volume of heptane to remove free fatty acids. A sample (0.1 ml) of the methanolic Hill's solution containing the acyl hydroxamate-*1*-^{14}C-$FeCl_3$ complex was added to a counting vial containing dioxane scintillation mixture and counted. No appreciable quenching was observed under these conditions.

Acyl-CoA dehydrogenase. The assay was essentially that described by Green et al. (5). A typical assay mixture consisted of: palmityl-CoA, 100 nmoles; glycyl glycine (*p*H 8.2), 20 μmoles; pyocyanine, 0.08 mg; triphenyl tetrazolium chloride, 1.2 mg; bovine serum albumin, 0.14 mg; protein, 2 or 4 mg; and water to a final volume of 0.6 ml. A control incubation without palmityl-CoA was performed for each enzyme level. The reaction tubes were placed in a vacuum desiccator and evacuated for 5 min at approximately 5 mm of Hg. The desiccator and its contents were incubated at 37 C for 1 hr. Air was then slowly admitted, and the reaction was terminated by the addition of 0.05 ml of 6 N HCl to each tube. The red-colored formazan that formed during the reaction was extracted from the aqueous phase with 6 ml of a mixture of acetone and carbon tetrachloride (1:3). The amount of formazan was determined by measuring the optical density of the organic phase at 485 nm. All values were corrected against those obtained for the control incubations.

β-Hydroxy acyl-CoA dehydrogenase. The assay used was a modification of the method of Wakil et al. (18). A typical mixture consisted of: Tris-chloride buffer (*p*H 7.0), 20 μmoles; acetoacetyl CoA, 50 nmoles; reduced NAD (NADH), 100 nmoles; supernatant protein, 10 to 300 μg; and water to 0.4 ml. Reactions were initiated by the addition of the enzyme and were assayed at 25 C. The reaction was followed by determining the rate of oxidation of NADH as measured by the decrease in absorbancy at 340 nm with the use of a Gilford model 2000 spectrophotometer. All values were corrected against those obtained with control incubations without substrate.

Enoyl-CoA hydrase. The assay was adapted from the procedure described by Mahler and Wakil (19). A typical assay mixture consisted of: 2-amino-2-methyl-1,3-propane diol (*p*H 9.0), 20 μmoles; mercaptoethanol, 2 μmoles; NAD, 0.4 μmole; pig heart β-hydroxyacyl-CoA dehydrogenase, 4 μg; crotonyl CoA, 100 nmoles; supernatant protein, 2 to 100 μg; and water to a final volume 0.4 ml. Reaction mixtures were preincubated for 2 min at 25 C in the absence of supernatant protein. The reaction was then initiated by the addition of supernatant protein, and the formation of NADH was followed by the increase in absorbancy at 340 nm with the use of a Gilford spectrophotometer.

Thiolase. The method used was a modification of that described by Stern (17). A typical assay mixture consisted of Tris-chloride buffer (*p*H 8.1), 80 μmoles; $MgCl_2$, 2 μmoles; CoA, 100 nmoles; mercaptoethanol, 2 μmoles; acetoacetyl CoA, 20 nmoles; supernatant protein, 20 to 600 μg; and water to 0.4 ml. Reactions were initiated by the addition of the enzyme. The cleavage of acetoacetyl CoA was measured by monitoring continuously the disappearance of absorbancy at 303 nm with the use of Gilford recording spectrophotometer. All values were corrected against those obtained with control incubations without enzyme.

RESULTS

Growth of *E. coli* on fatty acid. The ability of *E. coli* to grow on various fatty acids as sole carbon source was tested, and the results are summarized in Table 1. Oleate, palmitate, and myristate supported growth, whereas laurate, decanoate, octanoate, hexanoate, and butyrate did not. The doubling time on palmitate was about 3 hr, compared with 65 min on the amino acid medium. Growth was demonstrable on the long-chain fatty acids only after a distinct lag phase. However, growth on long-chain fatty acid was immediate when the inoculum consisted of cells grown on palmitate. These results indicated that an induction period was necessary before the fatty acids could be utilized.

To determine whether adapted cells would grow on shorter-chain fatty acids, cells were pregrown on palmitate and then transferred to decanoate. Growth was immediately observed but persisted for only 1 doubling (Table 1, experiment II). Furthermore, palmitate-adapted cells grew to a slightly greater extent on a medium containing both decanoate (1 g/liter) and palmitate (100 mg/liter), than on a medium containing palmitate (100 mg/liter) alone. These results suggested that decanoate could support growth as long as palmitate-induced enzyme levels were maintained; the cessation of growth after only one doubling indicated a dilution of these enzyme levels.

TABLE 1. *Growth characteristics of E. coli on fatty acid*

Expt no.[a]	Carbon source[b]	Lag phase (hr)	Extent of growth (no. of doublings)
I	Amino acid	0	4
	Butyrate	—	0
	Hexanoate	—	0
	Octanoate	—	0
	Decanoate	—[c]	0[c]
	Laurate	—[c]	0[c]
	Myristate	<10	4
	Palmitate	<10	4
	Oleate	<10	4
II	Amino acid	0	4
	Palmitate	0	4
	Decanoate	0	1
	Decanoate + palmitate (0.01%)	0	3
	Palmitate (0.01%)	0	2–3

[a] In experiment I, the inoculum was prepared by growing cells on amino acid as sole carbon source. In experiment II, the inoculum was prepared by growing cells on palmitate as sole carbon source. Sufficient inoculum of cells was added to produce a turbidity of 10 to 20 Klett units.

[b] The quantity of the carbon source in the growth media was as described in Materials and Methods, except where otherwise indicated.

[c] Prolonged incubation of these cultures led to growth due to the spontaneous formation of mutants.

Oxidation of fatty acids by resting cells. Resting cells grown on various carbon sources oxidized acetate, decanoate, and palmitate at different rates (Table 2). The rate of acetate oxidation was higher than that of either decanoate or palmitate, indicating that the terminal oxidation of acetyl CoA to CO_2 was not limiting the rate of fatty acid degradation. Furthermore, the oxidation of acetate by cells grown on palmitate was unchanged, whereas the rate of oxidation of decanoate and palmitate was considerably increased, indicating that the enzymes of β-oxidation had been induced. Cells grown on palmitate oxidized both palmitate and decanoate at rates eight times faster than cells grown on amino acid medium (Table 2). The increase in the rates of palmitate and decanoate oxidation is low compared to increases observed with some inducible systems (14), indicating a high uninduced level of the enzymes of β-oxidation. However, these uninduced levels were insufficient to maintain cell growth on fatty acid, since a distinct lag phase (cf. Table 1) was necessary to induce the enzymes of fatty acid oxidation to levels high enough to support growth. The inclusion of palmitate in the amino acid medium significantly increased the rates of fatty acid oxidation (Table 2), whereas the inclusion of decanoate or acetate had no effect, indicating that neither decanoate nor acetate induced fatty acid oxidation. Cells grown on a combined medium of acetate and amino

TABLE 2. *Oxidation of fatty acid by resting cells of E. coli grown on various carbon sources*

Carbon source	Fatty acid oxidation (nmoles per min per mg of cells, dry wt)		
	Palmitate	Decanoate	Acetate
Amino acid	0.17	0.31	91.8
Amino acid + glucose	0.03	0.04	0.13
Amino acid + acetate	0.12	0.36	38.0
Amino acid + decanoate	0.13	0.22	—
Amino acid + palmitate	1.02	1.32	87.0
Amino acid + palmitate + glucose	0.07	—	—
Palmitate	1.46	2.18	—

acid oxidized acetate only 40% as well as cells grown on amino acid alone. The addition of glucose to the amino acid medium yielded cells that oxidized palmitate, decanoate, and acetate at considerably reduced rates, and this glucose repression of fatty acid oxidation was observed even in the presence of palmitate in the growth medium. The repressive effects of glucose and acetate are probably explicable in terms of catabolite repression (10).

Oxidation of fatty acid by extracts of *E. coli*. Cell-free extracts of *E. coli* oxidized palmitate to CO_2 in the presence of various cofactors (Table 3). Very little oxidation was obtained in the absence of either CoA or ATP, and NAD and succinate were required for optimal rates. The succinate requirement suggested that the conversion of acetyl CoA to CO_2 involved the enzymes of the tricarboxylic acid cycle.

The oxidative activities of extracts prepared from cells grown on different carbon sources varied (Table 4). The degree of variation was comparable to that obtained with resting cells. Extracts obtained from cells grown in the presence of palmitate had higher oxidative activity than extracts of cells grown in the absence of palmitate. Furthermore, the presence of glucose in the growth medium completely repressed palmitate oxidation.

Induction of the enzymes of β-oxidation. To determine which of the enzymes of the β oxidation sequence were being induced, cell-free extracts were assayed for each enzyme. As shown in Table 5, the activities of all five enzymes (palmityl-CoA synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydrase, β-hydroxyacyl-CoA dehydrogenase, and thiolase) were induced. Specific activities of the enzymes prepared from cells grown on either palmitate or oleate as the sole carbon source were the highest, about seven times higher than those obtained from cells grown on amino acid medium. The extent of induction of the enzymes was essentially the same with either palmitate or oleate. The enzyme activities of the extracts of cells grown on amino acid medium was relatively high, but was further induced by the addition of palmitate. However, the addition of decanoate to the amino acid medium did not cause any increase in enzyme activities, indicating that decanoate did not induce these enzymes.

The addition of glucose to the amino acid growth medium yielded cells whose extracts had depressed levels of oxidative enzymes (Table 5), thus demonstrating that catabolite repression was expressed on all of the β-oxidation enzymes. Growth on glycerol had a slight repressive effect, as evidenced by the lower specific activity of the

TABLE 3. *Cofactor requirements for the oxidation of palmitate by extracts of E. coli*

Cofactor omitted	Palmitate oxidation (pmoles per min per mg of protein)
None	7.0
Succinate	4.1
NAD	1.9
CoA	0.1
ATP	0.1

TABLE 4. *Oxidation of palmitate by extracts of E. coli grown on various carbon sources*

Carbon source	Palmitate oxidation (pmoles per min per mg of protein)
Amino acid	8.3
Amino acid + acetate	8.0
Amino acid + palmitate	20.4
Amino acid + glucose	0

TABLE 5. *Specific activities of the enzymes of β-oxidation of E. coli grown on various carbon sources*

Carbon source	Enzyme specific activities[a] (nmoles per min per mg of protein)				
	Palmityl-CoA synthetase	Acyl-CoA dehydrogenase	Enoyl-CoA hydrase	β-Hydroxyacyl-CoA dehydrogenase	Thiolase
Amino acid	0.33	0.35	310	142	8.2
Amino acid less Triton X-100	—	—	285	115	5.5
Amino acid + glucose	0.03	0.09	20	7	0.8
Amino acid + decanoate	0.36	0.45	245	144	7.7
Amino acid + palmitate	0.69	0.57	726	344	20.0
Glycerol	0.20	—	164	108	5.4
Palmitate	1.83	1.43	2,075	1,072	77.0
Oleate	1.34	2.72	1,780	1,280	61.4

[a] Average values determined from several independent experiments.

enzymes than those obtained from cells grown on the amino acid medium. Since Triton X-100 was routinely added to growth media, it was important to determine whether it had any effect on the activities of the β-oxidation enzymes. As shown in Table 5, the specific activities of the enzymes extracted from cells grown on amino acids in the absence of Triton X-100 were comparable to those obtained from cells grown on similar medium which included Triton X-100.

When the various specific activities of enoyl-CoA hydrase, shown in Table 5, were plotted against the corresponding activities of the other four enzymes, a linear relationship was obtained (Fig. 1). This indicated that all five enzymes were coordinately induced and repressed over a wide range of activity and suggested some form of unit control. The differential plots obtained for the acyl-CoA dehydrogenase and palmityl-CoA ynthase, as determined by the method of least quares, did not pass through the origin. This may mean that these two enzymes are not truly coordinate, or may simply reflect the difficulties involved in assaying these two enzymes.

Uptake of decanoate-*1*-^{14}C. Although decanoate did not induce the enzymes of fatty acid oxidation (Tables 2 and 5), it was oxidized by resting cells more rapidly than palmitate. These results suggested that decanoate was transported into the cell. An attempt was made to determine the rate of decanoate uptake to demonstrate directly that its uptake was not a rate-limiting process and that the failure of this fatty acid to induce the enzymes of β-oxidation was not related to its inability to be internally accumulated at sufficient levels for induction.

The data in Fig. 2 demonstrated that decanoate uptake was a fairly rapid process. A steady state was attained after only 10 sec. The minimal rate of uptake, based on the 10-sec incubation time, was calculated as being about 15 nmoles of decanoate accumulated per min per mg (dry

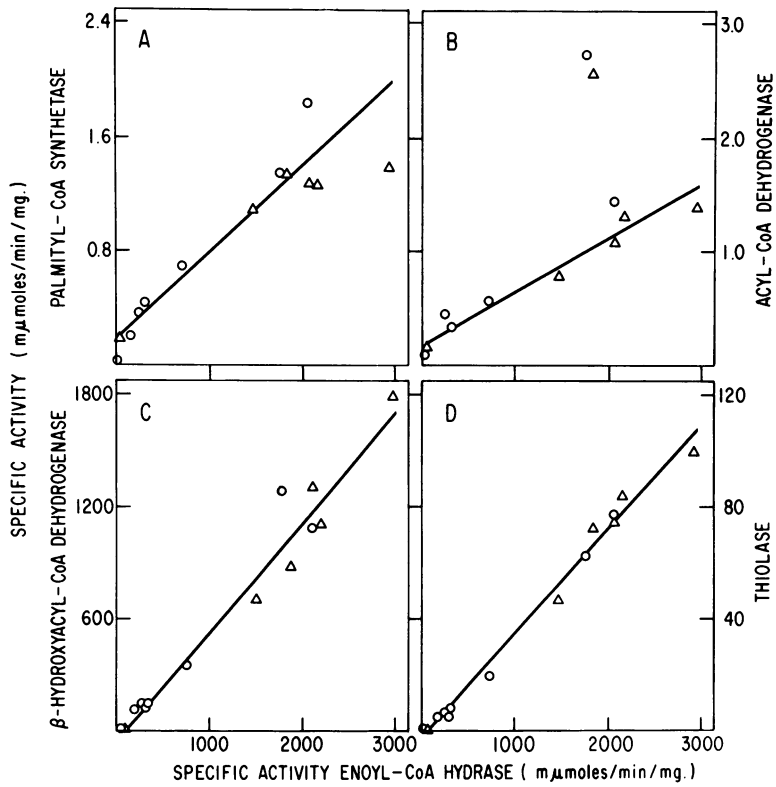

FIG. 1. *Differential plots of the specific activities of enoyl-CoA hydrase versus the specific activities of (A) palmityl-CoA synthetase, (B) acyl-CoA dehydrogenase, (C) β-hydroxyacyl-CoA dehydrogenase, and (D) thiolase. Values obtained for enzymes of the parent cells (○) were taken from Table 5. Values obtained for enzymes of the mutants D-1 and D-3 (△) were taken from Table 8. Lines of best fit were calculated by the method of least squares. The two high values for acyl dehydrogenase in Fig. 1B were not included in the calculation of least squares.*

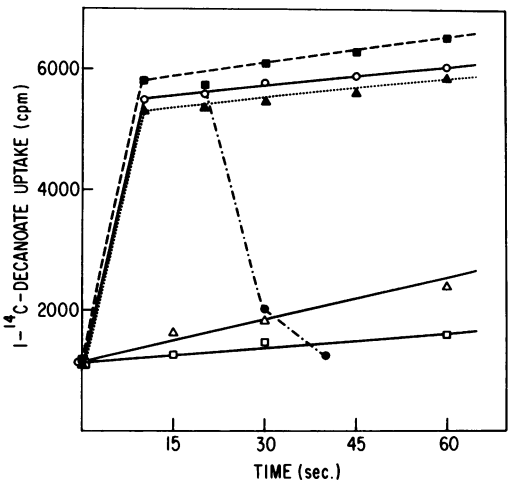

FIG. 2. *Uptake of decanoate-1-^{14}C by the parent strain and mutants D-1 and D-3. Reactions contained, in 0.5 ml of basic medium, 50 nmoles of decanoate-1-^{14}C (300,000 counts/min) and the following cells:* (○) *0.28 mg of parent strain cells;* (△) *0.28 mg of parent strain cells preincubated for 30 min with 2 × 10^{-3} M dinitrophenol;* (□) *0.28 mg of parent strain cells preincubated for 30 min with 10^{-3} M sodium azide;* (▲) *0.27 mg of mutant D-1 cells;* (■) *0.26 mg of mutant D-3 cells. For the "chase" experiment* (●), *0.28 mg of parent cells was incubated in 0.5 ml of basic medium with 50 nmoles (300,000 counts/min) of decanoate-1-^{14}C for 20 sec and was then diluted with 0.5 ml of 10^{-2} M decanoate in basic medium and further incubated for either 10 or 20 sec. All reactions were incubated at 37 C for the indicated times and terminated as described in Materials and Methods.*

weight). This rate was at least 40 times higher than the rate of decanoate oxidation by resting cells (Table 2), suggesting that decanoate uptake was, by no means, limiting the rate of its oxidation. The addition of 0.01 M decanoate to cells which had been incubated with decanoate-*1*-^{14}C for 20 sec, followed by 10 or 20 sec of incubation, resulted in very low uptake of decanoate-*1*-^{14}C, suggesting that the decanoate-*1*-^{14}C accumulated during the first 20 sec was removed by subsequent incubation with unlabeled decanoate (Fig. 2). When cells that had accumulated decanoate-*1*-^{14}C were filtered and then washed with 5 ml of ice-cold unlabeled decanoate (0.01 M in basic medium), the accumulated decanoate-*1*-^{14}C was almost completely removed (Table 6).

The uptake of decanoate was an energy-dependent process, as evidenced by its inhibition by either 0.01 M sodium azide or 0.01 M dinitrophenol (Fig. 2).

Growth of E. coli mutants on decanoate and laurate. As was iterated above, the strain of *E. coli* used for this study was unable to grow in a medium containing decanoate or laurate as sole carbon source. However, it was found that if medium containing decanoate or laurate as sole carbon source was inoculated with this organism growth did occur after prolonged incubation. The subsequent analysis of clones, derived from this type of culture, revealed that the property of these organisms that allowed them to grow on decanoate was attributable to a spontaneous mutation. The isolation mutants had the same auxotrophic requirements and phage sensitivity as the original strain.

Several mutants were isolated by this method, and the characteristics of two of these mutants, D-1 and D-3, were studied in some detail. Both mutants grew more slowly on amino acids than did the parent strain, and the extent of growth of the D-3 mutant was only half that observed for either the parent strain or the D-1 mutant. The D-3 mutant grew on both palmitate and decanoate without an apparent lag phase, even though the inoculum was grown on amino acid medium. The D-1 mutant, however, exhibited a lag of as much as 10 hr in a parallel experiment. Neither mutant grew on butyrate, hexanoate, or octanoate.

Oxidation of palmitate and decanoate by the D-1 and D-3 mutants. Resting cells of the D-1 and D-3 mutants, grown on amino acid media, oxidized palmitate and decanoate as shown in Table 7. Mutants D-1 and D-3 oxidized fatty acids at rates four and seven times greater, respectively, than those of the parent strain, indicating that these mutants possessed constitutive levels of the oxidative enzymes (cf. Table 7 and Table 2). The rate of oxidation by the D-1 mutant grown on palmitate or decanoate as sole carbon source was twice that of the same mutant grown on amino acid medium. In contrast, the rate of fatty acid oxidation by the D-3 mutant essentially remained the same whether the cells

TABLE 6. *Reversible uptake of decanoate-1-^{14}C by E. coli*

Time	Uptake of decanoate-*1*-^{14}C
sec	*counts/min*
0	1,200
20[a]	5,600
20[b]	1,230

[a]Cells were collected on a membrane filter and washed with 3 ml of ice-cold basic medium as described in Materials and Methods.

[b]Cells were collected on a membrane filter and washed with 5 ml of ice-cold decanoate (10^{-2} M in basic medium).

TABLE 7. *Oxidation of fatty acid by resting cells of D-1 and D-3 mutants of E. coli grown on various carbon sources*

Mutant	Carbon source	Fatty acid oxidized (nmoles per min per mg of cells, dry wt)	
		Palmitate	Decanoate
D-1	Amino acid	0.73	1.08
	Decanoate	1.60	2.50
	Palmitate	1.60	2.37
D-3	Amino acid	1.19	2.16
	Amino acid + glucose	0.17	0.06
	Decanoate	1.34	1.92
	Palmitate	1.46	2.06

were grown on palmitate, decanoate, or amino acid. The presence of glucose in the growth medium repressed the oxidation of fatty acids by the D-3 cells, an effect similar to that observed with the parent strain.

Activities of the β-oxidation enzymes of the D-1 and D-3 mutants. Cell-free extracts of both mutants were assayed for the various enzymes of β-oxidation. Growth of the mutants on amino acid yielded extracts with enzyme activities considerably higher than those of extracts of similarly grown parent strain cells (cf. Table 8 and Table 5). The specific activities of the enzymes of the D-3 mutants were on the average seven times higher than those of the parent strain, whereas the enzyme activities of the D-1 mutants were only five times higher than those of the parent strain. These relative activities were consistent with those obtained for the resting cells. Moreover, the enzyme levels of the extracts of the D-3 mutant were not appreciably induced by palmitate or decanoate, whereas the corresponding enzyme levels of the D-1 mutant were doubled when cells were grown on decanoate (Table 8). The presence of glucose in the growth medium repressed all the enzymes of β-oxidation of the D-3 mutants, again indicating that glucose was exerting a catabolite repression effect.

The individual enzyme activities were coordinately repressed and induced as shown in Fig. 1, again indicating that some form of coordinate control was effected.

Inability of the short-chain fatty acids to support growth of the D-1 and D-3 mutants. The fact that both mutants possessed constitutive levels of the enzymes of β-oxidation provided a reasonable explanation for the growth of these mutants on decanoate and laurate. However, like the parent strain, these mutants did not grow on short-chain fatty acids (butyrate, hexanoate, and octanoate) even after incubation for several weeks. It was found that butyrate and octanoate in concentrations of 1 g/liter inhibited the growth of *E. coli* on amino acids media, increasing the generation doubling time from 65 to 85 min, whereas the presence of hexanoate (1 g/liter) increased the generation time to over 4 hr.

Since it has been reported that α-amino butyrate and α-keto butyrate inhibit the synthesis of valine (M. Freundlich, J. S. MacDonald, and L. P. Clarke, Bacteriol. Proc., p. 113, 1967), the possibility was considered that the failure of the short-chain fatty acids to support growth was due to their inhibition of the synthesis of valine or some other essential metabolite. To circumvent this inhibitory effect, the short-chain fatty acid medium was supplemented with either Nutrient Broth or the amino acid mixture. However, no fatty acid-dependent growth was observed.

Uptake of decanoate-*1*-^{14}C by the D-1 and D-3 mutants. The uptake of decanoate-*1*-^{14}C by the D-1 and D-3 mutants was studied, and it was found that the uptake by both mutants was very similar to that of the parent strain (Fig. 2).

TABLE 8. *Specific activities of the enzymes of β-oxidation of the D-1 and D-3 mutants grown on various carbon sources*

Mutant	Carbon source	Enzyme specific activities (nmoles per min per mg of protein)				
		Palmityl-CoA synthetase	Acyl-CoA dehydrogenase	Enoyl-CoA hydrase	β-Hydroxyacyl-CoA dehydrogenase	Thiolase
D-1	Amino acid	1.08	0.77	1480	694	46.8
	Decanoate	1.46	1.37	2960	1770	99.3
D-3	Amino acid	1.33	2.55	1850	870	70.2
	Amino acid + glucose	0.18	0.16	48	2	0.3
	Decanoate	1.27	1.07	2090	1295	74.0
	Palmitate	1.26	1.30	2180	1095	81.8

DISCUSSION

A distinct lag phase was observed before growth of *E. coli* occurred on long-chain fatty acids. The lag phase was eliminated if the inoculum was grown on palmitate, suggesting that the lag phase was necessary for the induction of the enzymes of β-oxidation. Palmitate-grown cells oxidized fatty acids considerably faster than cells grown on amino acids or glucose, also suggesting that the levels of the enzymes of β-oxidation were induced in the fatty acid-grown cells. Assays of the key enzymes of β-oxidation in cell free extracts of cells grown on fatty acids confirmed that the levels of all five enzymes were significantly higher than those obtained in extracts of cells grown in absence of fatty acids (Table 5). Analyses of the relative changes of the specific activities of five enzymes of β-oxidation indicated that they were under some form of unit control (Fig. 1). The nature of the controlling factor(s) in this system and the target of its action is not known at present, but the type of induction expressed by this system is probably explained in terms of the model proposed by Jacob and Monod (7) for the induction of the *lac* operon.

The induction of the enzymes of β-oxidation appeared to be dependent upon the chain length of the inducing fatty acids. Long-chain fatty acids (myristate, palmitate, oleate) readily supported growth of *E. coli*. In contrast, shorter-chain acids (laurate, decanoate, octanoate, hexanoate, and butyrate) failed to support any growth, although these acids are readily oxidized by resting cells. The failure of the cells to grow on laurate and decanoate could be explained by the inability of these acids to induce the enzymes of β-oxidation. Palmitate-induced cells exhibited limited growth when transferred to medium containing decanoate as the sole carbon source, indicating that decanoate was able to support growth as long as induced enzyme levels were maintained. The early cessation of growth was probably a consequence of the dilution of these high enzyme levels because of cell growth in the absence of inducer. The inability of decanoate to induce the enzymes of fatty acid oxidation cannot be explained by a failure of cells to accumulate decanoate, since uninduced, resting cells of *E. coli* rapidly transported decanoate. This transport was energy-dependent and led to the intracellular accumulation of decanoate. However, if the induction model proposed for the *lac* operon is applied to the β-oxidation system, then one would expect long-chain fatty acids to combine with the product of the *i* gene (the repressor protein), rendering it inactive and thus allowing the operator gene to express itself; conversely, the repressor protein may not effectively interact with decanoate. The failure of short-chain acids ($<C_8$) to support growth cannot be explained in these terms, because the constitutive mutants, D-1 and D-3, did not grow on these acids. The nature of the active form of the inducing fatty acid is not known at present; it may be the free acid, its CoA or acyl carrier protein (11, 20) derivative, or some other unknown form.

Two hitherto undescribed strains, D-1 and D-3, were isolated as spontaneous mutants. These mutants were constitutive for the enzymes of β-oxidation and were able to grow on decanoate and laurate as sole carbon source. The activity of the enzymes of β-oxidation was coordinate with the activity of the enzymes of the parent strain (Fig. 1). Because of the manner in which these strains were obtained, they probably contain mutations which are single events affecting either the operator region or the repressor gene. A mutation in either gene would result in the constitutive synthesis of the enzymes of β-oxidation. To differentiate between these two alternatives, analysis of the linkage of the mutation to the structural genes for the oxidative enzymes, or a *cis-trans* dominace analysis, will be required. The uninduced enzyme activities of the mutant D-1 were significantly lower than those of either the mutant D-3 or the induced parent strain. These levels were induced not only by palmitate but also by decanoate. This observation can also be explained by a single-step mutation, since a mutation of the repressor might result in an altered specificity such that its affinity for decanoate is increased. Alternatively, a mutation in the operator region may have reduced its affinity for repressor such that decanoate can effectively compete with the operator region for the binding of the repressor. The possibility must not be overlooked that the induction of fatty acid oxidation may also be explained according to a positively controlled system, such as that described by Engelsberg and his colleagues (16) for the metabolism of arabinose in *E. coli*.

Recently, Overath et al. (13) reported the induction of the enzymes of fatty acid oxidation in a strain of *E. coli* K-12. These workers showed that extracts of cells grown on oleate had enzyme levels considerably higher than those found in extracts of cells grown in absence of oleate. They also found that the levels of the enzymes enoyl-CoA hydrase, β-hydroxyacyl-CoA dehydrogenase, and thiolase varied proportionately, and they concluded that these enzymes were coordinately controlled. The degree of induction reported by Overath and co-workers was somewhat higher than those reported here, possibly because of the differences between the strains employed.

The relatively low magnitude of induction indicated in the present study connoted a high uninduced level of the oxidative enzymes, which was nevertheless insufficient to yield immediate bacterial growth on fatty acids as the sole carbon source. The high uninduced level may be important in removing any toxic fatty acids that may be formed or as a control mechanism for the overall synthesis of lipid. Also, the high basal level of the oxidative enzymes may be the consequence of an "internal induction" by the products of the fatty acid-synthesizing system. It is of interest to point out that the mutants with constitutive levels of β-oxidation may be the consequence of the uncontrolled operation of the fatty acid biosynthetic pathway. This point is presently being investigated.

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ADDENDUM IN PROOF

It has been shown recently that cyclic adenosine-3', 5'- monophosphate (cyclic AMP) reverses the effect of glucose on the synthesis of β-galactosidase in *E. coli* (R. L. Perlman and I. Pastan, J. Biol. Chem., **243**:5420, 1968). We found that the glucose repression of fatty acid oxidation is partially reversed by growing cells in the presence of 10^{-3} M cyclic AMP. The rate of palmitate oxidation by cells grown on palmitate as the sole carbon source was 1.77 nmoles per min per mg (dry wt; Table 2), whereas the rate for cells grown on media containing both palmitate and glucose was only 0.14 nmoles per min per mg (dry wt). The addition of cyclic AMP (10^{-3} M) to the media containing both glucose and palmitate partially restored the cellular fatty acid oxidation rates (0.66 nmoles per min per mg). The addition of cyclic AMP to growth media which contained palmitate as the sole carbon source caused only a slight stimulation of the cellular rates of fatty acid oxidation (2.46 nmoles per min per mg), suggesting that the stimulating effect of cyclic AMP was due to a reversal of catabolite repression rather than to a direct activation of fatty acid oxidation.

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