*FATTY ACID MUTANT OF E. COLI LACKING A β-HYDROXYDECANOYL THIOESTER DEHYDRASE**

By David F. Silbert† and P. R. Vagelos

DEPARTMENT OF BIOLOGICAL CHEMISTRY, WASHINGTON UNIVERSITY SCHOOL OF MEDICINE, ST. LOUIS, MISSOURI

Communicated by Oliver H. Lowry, August 14, 1967

Fifteen to thirty per cent of the dry weight of bacterial membrane is lipid.[1] The other large fraction is protein. Essentially all of the lipid in *E. coli* is in the cell envelope. The lipid composition is generally characteristic of the bacterial family.[2] In *E. coli* the lipid is predominantly phosphatidyl ethanolamine.[3] The fatty acid composition of this phosphatidyl ethanolamine is markedly dependent on the age of the culture, temperature, and the composition of the medium.[2, 4] The availability of fatty acid biosynthetic mutants would permit a systematic examination of the role of fatty acids and their lipid derivatives in membrane function without variations in composition introduced by the above physiological factors. Thus, mutants unable to synthesize their own fatty acids could be supported on a defined source of natural fatty acids or on suitable analogues of these which would be incorporated into the cell membranes.

In the present report the isolation of an unsaturated fatty acid-requiring mutant from *E. coli* K12 and characterization of the enzymatic defect are described. The specific requirement of this organism for unsaturated fatty acids and the replacement of this requirement by various analogues will be the subject of a subsequent paper.

Materials.—An Hfr mutant of *E. coli*, strain K12, which requires pantothenate and thiamine was obtained from F. Jacob. *E. coli* K12 harvested in mid-log phase from enriched medium was purchased from Grain Processing Corporation. N-methyl-N′-nitro-N-nitrosoguanidine was a product of Aldrich Chemical Company. Oleic, palmitoleic, and elaidic acids, all 99+%, were obtained from Applied Science Laboratories. *cis*-Vaccenic acid, 99%, was available through the Hormel Institute. 9,10-methylene octadecanoate was a gift of J. Law. Tween 40 was supplied by Atlas Chemical Industries. Acetyl CoA and 2-C^{14}-malonyl CoA were synthesized as described previously.[5,6] D(−)β-hydroxydecanoate was isolated from stationary culture fluid of *Pseudomonas aeruginosa*[7] and D(−)β-hydroxydecanoyl-NAC[8] was prepared by the mixed anhydride method.[9] Precoated thin-layer plates containing 10% $AgNO_3$ in silica gel were supplied by Analtech, Inc. Glucose-6-phosphate dehydrogenase, Type VI, was purchased from Sigma Chemical Company.

Methods.—Cultures were grown at 37°C in a New Brunswick shaker, model G-25, in medium E[10] containing 0.2% carbon source, 2×10^{-6} *M* pantothenate, and 1.7×10^{-6} *M* thiamine. When a fatty acid was used as carbon source, it was transferred sterilely from a 10% stock solution of the potassium salt in water to the medium containing 0.5% Tween 40 and 0.00001% yeast extract. When a fatty acid was used as a supplement for an auxotroph, it was present at 0.005%.

For the isolation of mutants, Hfr 139 was grown on oleate as carbon source (to assure that sufficient fatty acid entered the cell) and treated with nitrosoguanidine according to the method of Adelberg.[11] Fatty acid auxotrophs were then obtained by means of penicillin enrichment[12] with succinate as carbon source and replica plating[13] on selective media. Mutant subcultures used in the present studies contained less than 0.1% spontaneous revertants.

Lipids were extracted from whole cells by the method of Folch *et al.*[14] Fatty acids were released from lipids by hydrolysis with 15% KOH in methanol at 70°C for 1 hr. For esterification 2% H_2SO_4 in methanol at 70°C for 1 hr was employed. GLC was performed at 175°C in an F and M model 402 instrument with a 6-ft glass column containing 10% diethylene glycol succinate on

chromasorb W. Flow rates of helium, hydrogen, and compressed air were 30, 25, and 225 ml/min, respectively.

Crude cell extracts buffered in 0.01 *M* potassium phosphate, pH 7.0, containing 0.01 *M* β-mercaptoethanol were prepared at 0°C in a French pressure cell. Cellular debris was removed by centrifugation at 40,000 × *g* for 45 min.

Enzyme assays: Fatty acid synthetase: Fatty acid synthesis was measured *in vitro* in a reaction mixture (0.50 ml) containing 50 μmoles potassium phosphate, pH 7.0, 1 μmole β-mercaptoethanol, 15 mμmoles acetyl CoA, 30 mμmoles 2-C^{14}-malonyl CoA (1.0 μc/μmole), 0.75 μmole TPN^+, 2,5 μmoles glucose-6-phosphate, 0.65 unit of glucose-6-phosphate dehydrogenase (130 units/mg). and 24 μg of freshly reduced ACP.[15] The reaction was started by the addition of 0–0.5 mg of protein from crude extracts and incubated at 37°C for 30 min. It was stopped with 1.0 ml of 15% KOH in methanol. Long-chain fatty acids were recovered by saponification as described above, acidification of the product, and extraction into petroleum ether. Aliquots were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3375. The assay was linear over the range studied. About 30% of the 2-C^{14}-malonyl CoA was incorporated into long-chain fatty acids. Essentially no radioactivity from the blanks (0 mg of protein) appeared in the petroleum ether phase. For the identification of unsaturated and saturated fatty acids in the reaction product unlabeled carrier fatty acids were added, and the free acids were converted to methyl esters and chromatographed on a TLC plate of 10% $AgNO_3$ in silica gel using *n*-hexane:-diethyl ether (9:1).[16] The fatty acid ester spots were identified under UV light after spraying the plate with 0.2% dichlorofluorescein. R_f values for polar, unsaturated, and saturated fatty acid esters were 0.08, 0.45, and 0.54, respectively. Radioactivity recovered from the plates was consistently 80% of that spotted.

Spectrophotometric assay of trans-α,β-decenoyl-NAC formation from β-hydroxydecanoyl-NAC: The reaction mixture (0.2 ml) contained 54 mμmoles D(−)β-hydroxydecanoyl-NAC and 2 μmoles potassium phosphate, pH 7.0. The enzyme preparation was added with stirring and the increase in absorbance at 263 mμ associated with the formation of a *trans-α,β* unsaturated thioester was followed at 25°C. Initial rates were taken as a measure of α,β dehydration activity. One unit is defined as that amount of enzyme catalyzing the formation of one mμmole of *trans-α,β*-decenoyl-NAC per minute. The equivalent absorbance change in 0.2 ml is 0.0335/min (E = 6700).

Results.—Growth properties of the mutant: Mutants in *E. coli* requiring unsaturated fatty acids have been isolated with a frequency of about 0.3% after one penicillin cycling. The mutant for which the enzymatic defect is defined in this paper does not grow on glucose, glycerol, acetate, or succinate. It can grow on oleate as a carbon source with the same rate as the wild type. Alternately, it can grow on glycerol if the growth medium is supplemented with an appropriate unsaturated fatty acid. For example, approximately 2×10^{-4} *M* oleate as supplement permits full growth on glycerol. Although a number of unsaturated fatty acids and their analogues are suitable supplements, branched-chain, saturated, and hydroxy fatty acids do not replace this requirement.

Fatty acid content of mutant organism: The second evidence of the character of the block is the finding that the composition of unsaturated fatty acids in the mutant, unlike that of the wild type, reflects the unsaturated fatty acid provided in the medium. The lipids of the wild-type strain (Fig. 1*A*) contain C_{16} and C_{18} monoenoic acids and their cyclopropane derivatives, while those of the mutant contain only the unsaturated fatty acid provided in the medium together with its cyclopropane derivative. For example, in the mutant grown with palmitoleate (Fig. 1*B*), there is hexadecenoate and the C_{17} cyclopropane derivative but no octadecenoate or C_{19} cyclopropane derivative. In the mutant grown with oleate (Fig. 1*C*), there is octadecenoate and C_{19} cyclopropane fatty acid. There is also hexadecenoate here, but this probably represents a delta-seven derivative which is formed by removal of two carbons from the carboxyl terminus of the exogenous

oleate. This conclusion is suggested by the following observations: (1) C_{17} cyclopropane fatty acid is not formed as in the wild type, indicating that the C_{16} monoenoic peak in question is not a suitable substrate for this conversion; (2) when C^{14}-acetate is added to the medium it is incorporated into hexadecenoate of the wild type but not of the mutant organism; (3) hexadecenoate is not formed when

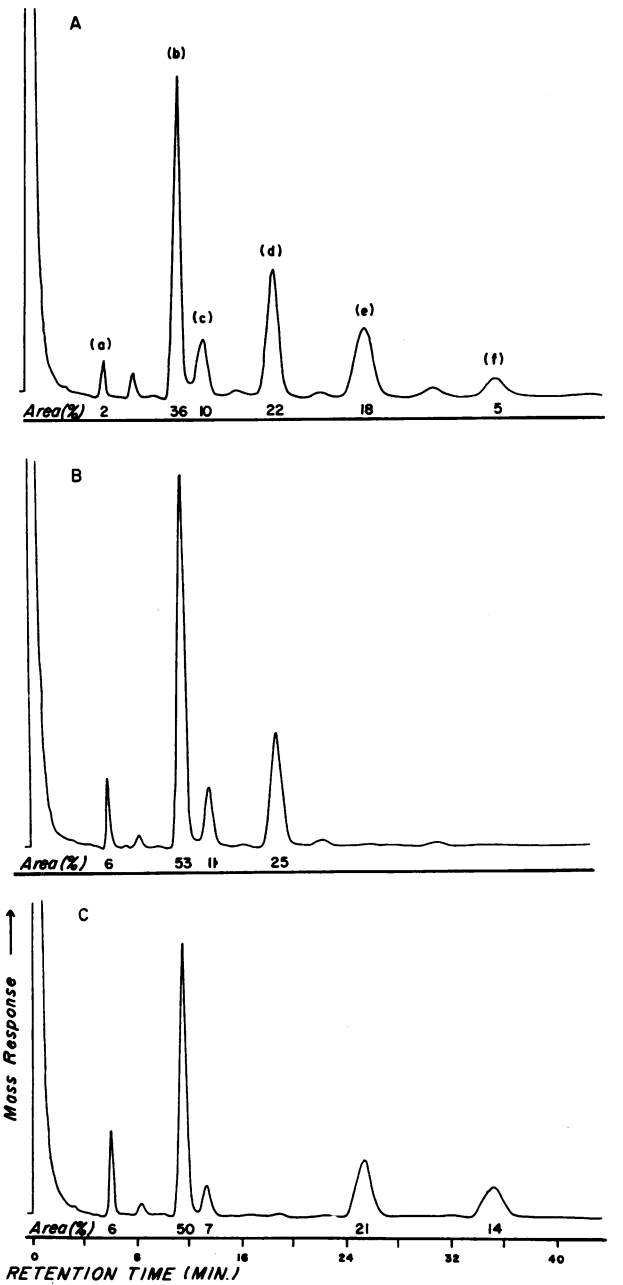

FIG. 1.—GLC of fatty acid methyl esters derived from stationary cultures of wild-type and mutant strains. The procedure is described in *Methods*.

(*A*) Fatty acid esters from wild type grown on glycerol and oleate;

(*B*) esters from the mutant grown on glycerol and palmitoleate;

(*C*) esters from the mutant grown on glycerol and oleate.

The peaks are identified as follows: (*a*) tetradecanoate, (*b*) hexadecanoate, (*c*) hexadecenoate, (*d*) 9,10-methylene hexadecanoate, (*e*) octadecenoate, (*f*) 11,12-methylene or 9,10-methylene octadecanoate. For each major peak, the corresponding contribution to total fatty acids is recorded in %.

TABLE 1

TLC DISTRIBUTION OF INCORPORATED COUNTS (%)

	Wild type (from glycerol-oleate)	Mutant (from glycerol-oleate)	Mutant (from glycerol-palmitoleate)
Saturated fatty acids	67.9	93.9	95.5
Unsaturated fatty acids	27.2	0.4	1.5
Hydroxy fatty acids	1.4	1.9	1.8

Wild-type and mutant strains were grown on media described in *Methods* and containing in addition sodium acetate-1-C^{14} at a concentration of 2.44×10^{-6} *M* and of specific activity 29 $\mu c/\mu$mole. Six % of the acetate from the medium appeared in the wild-type fatty acids and 2% appeared in the mutant fatty acids. The number of cpm chromatographed were 12,500, 5,400, and 6,800 for wild type, mutant (glycerol-oleate), and (glycerol-palmitoleate), respectively.

9,10-methylene octadecanoate serves as supplement but is formed when octadecenoates other than oleate such as vaccenate or elaidate are utilized.

In vivo fatty acid synthesis: The third form of evidence for the enzymatic defect is the demonstration that the mutant does not synthesize any unsaturated fatty acids from acetate. Table 1 shows the distribution of radioactivity derived from tracer amounts of C^{14}-acetate in the medium and incorporated into the saturated, unsaturated, and hydroxy fatty acids of the wild-type and mutant organisms. Insignificant radioactivity is associated with the unsaturated fatty acid fraction from the mutant organism grown in medium supplemented with oleate or palmitoleate while 27% of the radioactivity which is found in fatty acids from the wild-type strain is in this fraction. These findings were confirmed by GLC of the eluted material which showed radioactivity in hexadecenoate and octadecenoate from the wild type but not the mutant.

Since the cyclopropane fatty acids migrate with the saturated fatty acids on the TLC, the saturated fraction was also eluted and fractionated on GLC. The specific radioactivity was determined for the C_{16} saturated and the cyclopropane fatty acids (Table 2). As anticipated, no radioactivity was found in cyclopropane derivatives isolated from the lipids of the mutant strain. The cyclopropane derivatives which are synthesized *de novo* in the wild-type strain were isolated as a control and shown to have specific activities comparable to that of the saturated fatty acid.

Similar findings were obtained when wild-type and mutant organisms were grown on oleate as sole carbon source. That is, the wild type continues to make unsaturated fatty acids as demonstrated by the incorporation of C^{14}-acetate; whereas the mutant derives its unsaturated fatty acids completely from the medium.

In vitro fatty acid synthesis: *In vitro* studies confirmed the *in vivo* data, demonstrating the inability of mutant crude cell extracts to catalyze the synthesis of unsaturated fatty acid. Table 3 shows that 20% of the fatty acids synthesized

TABLE 2

INCORPORATION OF C^{14}-ACETATE INTO FATTY ACIDS

Source	Hexadecanoate (cpm)	Hexadecanoate (μg)	Hexadecanoate ($\mu c/\mu$mole)	C_{17} Cyclopropane (cpm)	C_{17} Cyclopropane (μg)	C_{17} Cyclopropane ($\mu c/\mu$mole)	C_{19} Cyclopropane (cpm)	C_{19} Cyclopropane (μg)	C_{19} Cyclopropane ($\mu c/\mu$mole)
Wild type (glycerol + oleate)	304	1.03	0.042	243	0.55	0.066	52	0.23	0.038
Mutant (glycerol + oleate)	450	1.69	0.038	—	—	—	7	0.92	0.001
Mutant (glycerol + palmitoleate)	464	2.75	0.024	1	1.01	0.000	—	—	—

The saturated fraction from the TLC shown in Table 1 was scraped from the plate and the methyl esters eluted with ethyl ether. Approximately 6000, 4000, and 5000 cpm of radioactivity from wild type, mutant (glycerol-oleate), and mutant (glycerol-palmitoleate), respectively, were injected into the GLC, and radioactive material associated with the major peaks was collected in Teflon tubing.

TABLE 3

In vitro FATTY ACID SYNTHESIS WITH CRUDE EXTRACTS

Extract	Saturated (%)	Unsaturated (%)	Polar (%)
Wild type	20	64	12
Mutant	77	3	18

Fatty acid synthetase assay is described under *Methods*. In the experiment represented here 29,160 cpm and 19,620 cpm were formed by 0.25 mg of crude protein from wild-type and mutant cells, respectively.

by wild-type extract is saturated and 64% unsaturated, while 77% of the product synthesized by mutant extract is saturated and only 3% unsaturated.

Correction of mutant synthesis by wild-type extract: Subsequently it was observed that small amounts of wild-type extract added to mutant extract dramatically corrected mutant unsaturated fatty acid synthesis. Furthermore, wild-type extract heated at 50°C for six minutes to destroy over-all fatty acid synthesis by the extract still corrected mutant unsaturated synthesis. The latter observations are shown in Figure 2. Increments of heated wild-type extract produced up to a twofold increase in mutant extract total fatty acid synthesis. This change corresponds to a shift from 3% unsaturated and 82% saturated fatty acids by mutant extract alone to 71% unsaturated and 26% saturated fatty acids with the addition of 0.2 mg heated wild-type protein. In this same experiment the control unheated wild-type extract formed 73% unsaturated and 21% saturated product. Furthermore, GLC of the unsaturated fatty acids in the "corrected" mutant product showed a distribution of 15% hexadecenoate and 76% octadecenoate, quite comparable to the wild-type unsaturated fatty acids which are composed of 11% hexadecenoate and 81% octadecenoate.

The lowest curve in Figure 2 shows that the heated wild-type extract did not catalyze fatty acid synthesis. Additional controls showed (1) that mutant extract heated at 50°C in the same fashion and added to untreated mutant extract failed to stimulate or correct unsaturated fatty acid synthesis, and (2) that wild-type extract heated to 100°C for 12 minutes is unable to alter mutant synthesis.

Identification of the enzymatic defect: The properties of the active component from the 50°C heated wild-type extract suggest the β-hydroxydecanoyl thioester dehydrase described by Norris *et al.*[17] and Kass *et al.*[18, 19] This enzyme catalyzes the interconversion of the thioesters of D(−)β-hydroxydecanoate, *trans*-α,β-

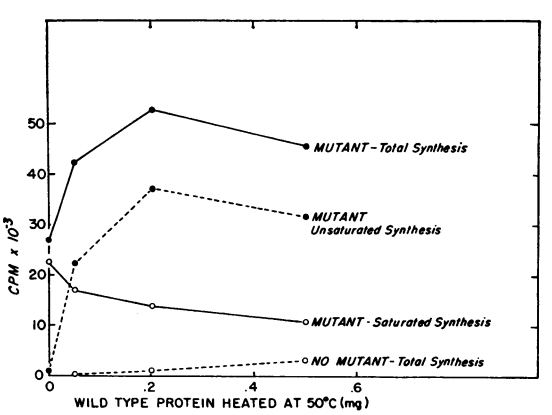

FIG. 2.—Correction of mutant synthesis by addition of heat-treated wild-type extract. Fatty acid synthesis and fractionation of the products are described in *Methods*. Protein (0.5 mg) from crude extract of mutant cells was used in these experiments where indicated. Heated wild-type protein was obtained by treating 1 ml of wild-type crude extract (25 mg/ml) at 50°C for 6 min. Aliquots of this supernatant fluid were added to the mutant extract as indicated.

TABLE 4

β-HYDROXYDECANOYL THIOESTER DEHYDRASE ACTIVITY

Protein source	Amount (mg)	Units of activity
Wild type	0.157	0.480
	0.314	1.010
Mutant		
Culture 1	0.116	0.030
	0.232	0.030
Culture 2		
Preparation 1	0.246	0.030
Preparation 2	0.318	0.048
Mutant (culture 1)	0.116	
plus	plus	0.480
Wild type	0.157	

Purification of the crude extracts included the protamine treatment, 50°C heating, and 0–70% ammonium sulfate precipitation described by Kass and Bloch[18] for the dehydrase preparation. Three mutant preparations were made from two separate cultures. The spectrophotometric assay of activity is given in *Methods*. One unit is mμmoles of *trans*-α,β-decenoyl thioester formed/min.

decenoate, and *cis*-β,γ-decenoate. Wild-type and mutant extracts were partially purified, essentially according to the procedures of Kass and Bloch,[18] and examined for the ability to catalyze the dehydration of β-hydroxydecanoyl thioester to form α,β-decenoyl thioester. This activity was present in preparations from wild-type cells but was missing in those from the mutant strain (Table 4). The combination of wild type and mutant preparations yielded activity comparable to the wild-type preparation alone. Thus, the inactivity of mutant extracts was not due to the presence of inhibitor or the inactivation of substrate. The catalytic activity of the wild-type extract was destroyed by boiling. When β-hydroxydecanoyl-ACP was used as substrate, the reaction rates were sixfold faster but there still was no activity in mutant extract.

Correction of mutant synthesis by partially purified dehydrase: If the β-hydroxydecanoyl thioester dehydrase is the site of the biosynthetic block, addition of the purified wild-type enzyme should specifically restore unsaturated fatty acid synthesis to the mutant extract. The enzyme was partially purified from crude cell extracts of *E. coli* K12. Equal units of dehydrase activity from several stages in the purification were shown to correct unsaturated synthesis by mutant extract to nearly the same extent (Fig. 3). Despite the complexity of this assay, including the lack of linearity over the range of dehydrase used, unsaturated synthesis is proportional to the amount of enzyme used and is nearly independent of the stage of purification for three different levels of enzyme. There is a relative purification of over 200-fold between the ammonium sulfate and the DEAE-cellulose steps. It seems unlikely that another enzyme is responsible for the correction of mutant synthesis and is carried together with the dehydrase through this extent of purification.

Discussion.—Unsaturated fatty acids have an essential role in most if not all organisms. Unsaturated fatty acid deficiencies have been produced in mammalian[20] and avian[21] species and unsaturated fatty acids have been shown to be growth requirements for certain microorganisms.[22–24] Although the wild-type forms do not require fatty acids, unsaturated fatty acid mutants have been derived from *Neurospora crassa*[25] and *Saccharomyces cerevisiae*.[26] The present studies describe the isolation and characterization of the first fatty acid biosynthetic mutant in bacteria. A major feature of the biosynthetic pathway for fatty acids

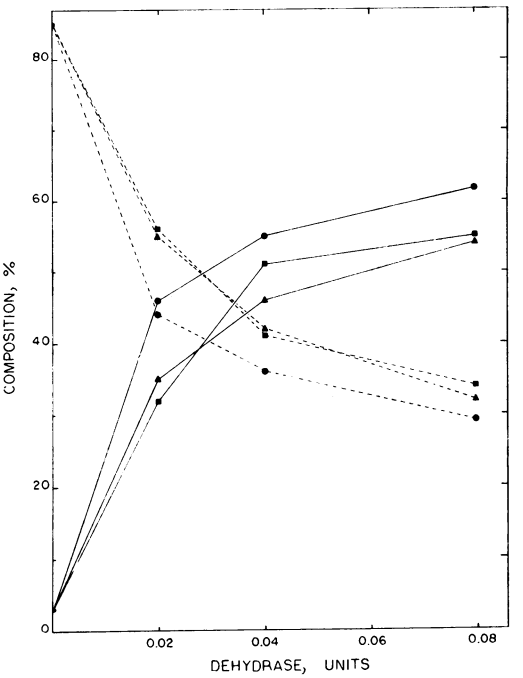

FIG. 3.—Restoration of unsaturated fatty acid synthesis by β-hydroxydecanoyl thioester dehydrase. Fatty acid synthesis and fractionation of product are described in *Methods*. Protein (0.35 mg) from mutant crude extract was used. β-Hydroxydecanoyl thioester dehydrase was purified according to the method of Kass and Bloch[18] from commercially grown *E. coli* K12 and added as indicated. The specific activity for various steps in the preparation are ammonium sulfate = 0.43 units/mg, G-100 Sephadex = 36.8 units/mg, and DEAE-cellulose = 90.5 units/mg. These three steps represent a relative purification of 1, 86, and 210, respectively.
Symbols: ——, unsaturated fatty acids;
- - - - -, saturated fatty acids;
●, ammonium sulfate step;
▲, G-100 sephadex step;
■, DEAE-cellulose step.

in *E. coli* and related species[27] is that they derive their unsaturated fatty acids from an intermediate chain length β-hydroxy fatty acid and are not able to obtain the unsaturated fatty acids from homologous long-chain saturated fatty acids by aerobic desaturation. *Neurospora* and *Saccharomyces* utilize this latter mechanism and thus the mutants isolated, although not fully characterized, must have defects in enzymes beyond the formation of long-chain saturated fatty acids.

The enzymatic defect in the *E. coli* mutant lies in the β-hydroxydecanoyl thioester dehydrase described by Bloch and co-workers.[17–19] This enzyme converts β-hydroxydecanoyl thioester to *cis*-3-decenoyl thioester and *trans*-2-decenoyl thioester, the latter product predominating. *cis*-3-Decenoyl-ACP is then elongated with the preservation of the *cis* double bond to C_{16} and C_{18} monoenoic fatty acids.[28] The present findings firmly establish that the β-hydroxydecanoyl thioester dehydrase is required by *E. coli* for unsaturated fatty acid synthesis. Furthermore, the finding that this mutant, which lacks this specific β-hydroxydecanoyl thioester dehydrase, synthesizes saturated fatty acids normally indicates that another enzyme exists which catalyzes α,β dehydration of β-hydroxydecanoyl-ACP to *trans*-2-decenoyl-ACP for the formation of long-chain saturated fatty acids. It is thought that this other enzymatic activity was lost in the partial purification of extracts which was necessary for the spectrophotometric assay at 263 mμ of β-hydroxydecanoyl-NAC dehydrase activity.

The mutant organism described in this paper should be useful in a number of studies including (1) the further elucidation of the enzymatic steps and their regulation in the fatty acid biosynthetic pathway, (2) the preparation of membranes with defined fatty acid composition for structural and functional studies, and (3)

the preparation of phospholipids of varying fatty acid composition (with or without radioactive label) for use in enzymatic studies.

Summary.—An unsaturated fatty acid mutant derived from *E. coli* K12 is described. The enzymatic defect is localized to a β-hydroxydecanoyl thioester dehydrase. The implication of these findings in the biosynthesis of fatty acids and in the study of bacterial membranes is discussed.

We are very much indebted to Dr. Luis Glaser for the rhamnolipid from *Pseudomonas aeruginosa*, to Dr. John Law for his suggestions concerning bacterial growth on fatty acids, and to Drs. Leon Kass and Konrad Bloch for making known to us the details of their purification of a β-hydroxydecanoyl thioester dehydrase from *E. coli*. The technical assistance of Mrs. Zita Zimbo and Mr. Jesse Watt in one phase of this work is gratefully acknowledged.

* Supported by grants NIH 1-R01-HE10406, NSF GB-5142X, and by a grant of the American Cancer Society to Washington University.

† Postdoctoral fellow of the American Cancer Society.

[1] Lennarz, W. J., *Advan. Lipid Res.*, **6**, 175 (1966).

[2] Kates, M., *Advan. Lipid Res.*, **2**, 17 (1964).

[3] Kaneshiro, T., and A. G. Marr, *J. Lipid Res.*, **2**, 184 (1962).

[4] Marr, A. G., and J. L. Ingraham, *J. Bacteriol.*, **84**, 1260 (1962).

[5] Simon, E. J., and D. Shemin, *J. Am. Chem. Soc.*, **75**, 2520 (1953).

[6] Trams, E. G., and R. O. Brady, *J. Am. Chem. Soc.*, **82**, 2972 (1960).

[7] Goldman, P., and P. R. Vagelos, *J. Biol. Chem.*, **236**, 2620 (1961).

[8] Jarvis, F. G., and M. J. Johnson, *J. Am. Chem. Soc.*, **71**, 4124 (1949).

[9] The abbreviations used are: acyl-NAC, acylthioester of N-acetyl cysteamine; TPN^+, triphosphopyridine nucleotide; ACP, acyl carrier protein; TLC, thin-layer chromatography; GLC, gas liquid chromatography; DEAE, diethylaminoethyl.

[10] Vogel, H. J., and D. M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).

[11] Adelberg, E. A., M. Mandel, and G. C. C. Chen, *Biochem. Biophys. Res. Commun.*, **18**, 788 (1965).

[12] Lubin, M., *J. Bacteriol.*, **83**, 696 (1962).

[13] Lederberg, J., and E. M. Lederberg, *J. Bacteriol.*, **63**, 399 (1952).

[14] Folch, J., M. Lees, and G. H. Sloane Stanley, *J. Biol. Chem.*, **226**, 497 (1957).

[15] Majerus, P. W., A. W. Alberts, and P. R. Vagelos, these PROCEEDINGS, **51**, 1231 (1964).

[16] Pugh, E. L., F. Sauer, M. Waite, R. E. Toomey, and S. J. Wakil, *J. Biol. Chem.*, **241**, 2635 (1966).

[17] Norris, A. T., S. Matsumura, and K. Bloch, *J. Biol. Chem.*, **239**, 3653 (1964).

[18] Kass, L. R., and K. Bloch, personal communication.

[19] Brock, D. T. H., L. R. Kass, and K. Bloch, *Federation Proc.*, **25**, 340 (1966).

[20] Burr, G. O., *Federation Proc.*, **1**, 224 (1942).

[21] Reiser, R., *J. Nutrition*, **42**, 319, 325 (1950).

[22] Shorb, M. S., and P. G. Lund, *J. Protozool.*, **6**, 122 (1959).

[23] Hutner, S. H., *J. Bacteriol.*, **43**, 629 (1942).

[24] Cohen, S., J. C. Snyder, and J. H. Mueller, *J. Bacteriol.*, **41**, 581 (1941).

[25] Lein, J., and P. Lein, *J. Bacteriol.*, **58**, 595 (1949).

[26] Resnick, M. A., and R. K. Mortimer, *J. Bacteriol.*, **92**, 597 (1966).

[27] Bloch, K., P. Baronowsky, H. Goldfine, W. J. Lennarz, R. Light, A. T. Norris, and G. Scheuerbrandt, *Federation Proc.*, **20**, 921 (1961).

[28] Nagai, J. and K. Bloch, *J. Biol. Chem.*, **242**, 357 (1967).