[Chem. Pharm. Bull. 30(7)2503—2511(1982)]

Studies on the Metabolism of Unsaturated Fatty Acids. VII.[1] Separation and General Properties of Reduced Nicotinamide Adenine Dinucleotide Phosphate-dependent *trans*-2-Enoyl-Coenzyme A Reductase from *Escherichia coli* K-12

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(Received, November 24, 1981)

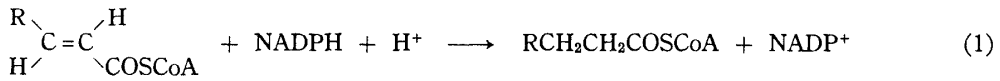
An enzyme fraction which catalyzes the reduction of *trans*-2-enoyl-coenzyme A (*trans*-2-enoyl-CoA) to the corresponding saturated acyl-CoA derivative was separated from *Escherichia coli* (*E. coli*) extracts.

The enzyme utilized reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a sole electron donor for the reduction of substrates, and it acted on acyl-CoA substrates, but not on acyl-carrier protein esters. Therefore, the name NADPH-dependent *trans*-2-enoyl-CoA reductase was proposed for this enzyme. The reductase was stable at around pH 7—8, and was active over a wide pH range between 6 and 9. It had a functional thiol group and was readily inhibited by thiol reagents such as *p*-hydroxymercuribenzoic acid, and by cupric and some other metal ions. The enzyme was induced when *E. coli* cells were cultured in a medium containing oleic acid.

These results suggest that this reductase does not participate in the *de novo* synthesis of fatty acids but is a new enzyme, although its physiological role is uncertain at the present stage.

Keywords——*trans*-2-octenoic acid; *trans*-2-[6,7,8-$^2H_7$]octenoic acid; *trans*-2-enoyl-CoA reductase; *Escherichia coli*; NADPH; chain elongation

A previous report from this laboratory has shown that an enzyme separated from *Escherichia coli* (*E. coli*) is capable of reducing *cis*-2-alkenoyl-coenzyme A (*cis*-2-alkenoyl-CoA) in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH).[2] Gel filtration of cell-free extracts of *E. coli* through a column of Sephacryl S-200 Superfine led to the elution of two active fractions with *cis*-2-octenoyl-CoA as a substrate (the decrease in absorbance at 340 nm was monitored). However, gas chromatography revealed that usual preparations of *cis*-2-alkenoyl-CoA contained considerable amounts of the corresponding *trans*-isomer formed by the interaction of the *cis*-substrate and reduced CoA.[3] The oxidation of NADPH was also observed upon addition of the first fraction in the presence of *trans*-2-octenoyl-CoA. Furthermore, when pure *cis*-2-octenoyl-CoA prepared according to our modified method[3] was employed, the consumption of NADPH was observed exclusively in the presence of the second fraction, indicating there were two different enzymes, referred to as NADPH-dependent *cis*-2-enoyl-CoA reductase and NADPH-dependent *trans*-2-enoyl-CoA reductase. The latter enzyme catalyzes the reduction of *trans*-2-alkenoyl-CoA in the presence of NADPH as shown in equation 1.[4]



$$\begin{matrix} R & & H \\ & C=C & \\ H & & COSCoA \end{matrix} + NADPH + H^+ \longrightarrow RCH_2CH_2COSCoA + NADP^+ \qquad (1)$$

On the other hand, two distinct reductases exist in cell-free extracts of *E. coli* as reported by Weeks and Wakil.[5] One is reduced nicotinamide adenine dinucleotide (NADH)-dependent enoyl-acyl-carrier protein (enoyl-ACP) reductase and the other is NADPH-dependent enoyl-ACP reductase, both of which catalyze the second reductive step in the sequence of reactions in fatty acid synthesis shown in equations 2 and 3 below:

$$\begin{matrix} \mathrm{R} & & \mathrm{H} \\ & \mathrm{C{=}C} & \\ \mathrm{H} & & \mathrm{COSR'} \end{matrix} + \mathrm{NADH} + \mathrm{H^+} \longrightarrow \mathrm{RCH_2CH_2COSR'} + \mathrm{NAD^+} \qquad (2)$$

$$\begin{matrix} \mathrm{R} & & \mathrm{H} \\ & \mathrm{C{=}C} & \\ \mathrm{H} & & \mathrm{COSACP} \end{matrix} + \mathrm{NADPH} + \mathrm{H^+} \longrightarrow \mathrm{RCH_2CH_2COSACP} + \mathrm{NADP^+} \qquad (3)$$

where R is H or an alkyl group, and R'SH is ACP or CoA. The NADH-dependent reductase utilizes both alkenoyl-ACP and alkenoyl -CoA substrates, whereas the NADPH-dependent reductase acts on the ACP esters but not CoA derivatives.[5]

This paper deals with the separation of NADPH-dependent *trans*-2-enoyl-CoA reductase from the two enoyl-ACP reductases, and with its general properties.

Materials and Methods

Fatty Acids——The *trans*-2-isomers of hexenoic, octenoic, decenoic, dodecenoic and tetradecenoic acids were synthesized by a Knoevenagel condensation of malonic acid with aldehydes in the presence of pyridine according to the report of Boxer and Linstead.[6] Homo-oleic (*cis*-10-nonadecenoic) acid was synthesized starting from oleic acid according to the reports of Klenk and Mohrhauer[7] and of Friedman and Schechter.[8] *trans*-2-[6,7,8-$^2H_7$]Octenoic acid was synthesized for this and other purposes, and the method will be reported elsewhere. These synthesized acids were purified by silicic acid column chromatography.[9] The purity of each acid was greater than 98%, as determined by gas chromatography. Oleic, linoleic and elaidic acids were purchased from Wako Pure Chemical Industries Ltd., (Osaka).

Other Chemicals——CoA, NADPH, and NADH were purchased from Kyowa Hakko Co. Ltd., (Tokyo). *E. coli* ACP was a generous gift from Prof. S.J. Wakil of Baylor College of Medicine, Houston, Texas, U.S.A. All other chemicals were of reagent grade and were obtained from usual commercial sources.

Preparation of Thiol Esters——The CoA derivatives of *trans*-2-alkenoic acids were prepared by the interaction of reduced CoA with the mixed anhydride derivatives of the alkenoates according to the method of Wieland and Koppe[10] except for crotonyl-CoA, which was prepared by the reaction of CoA and crotonic anhydride according to the report of Weeks and Wakil.[5] The ACP ester of *trans*-2-octenoic acid was synthesized and purified according to the report of Weeks and Wakil.[5]

Cultivation of *E. coli*——Cells of *E. coli* K-12 were grown in a liquid medium containing 1% meat extract and 1% peptone, and 1% glucose or 0.3% of one of the fatty acids (initial pH 7.2) at 37°C with vigorous shaking. The cells were collected at near the end of the logarithmic phase and washed with 0.01 M potassium phosphate buffer (pH 7.0).

Enzyme Preparations——All operations were performed at 0—4°C unless otherwise mentioned.

Crude Extracts: The collected cells were suspended in 2.4 volumes of 0.05 M potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid dipotassium salt (EDTA-2K), and sonicated for 10 min with a Tomy UR-200P ultrasonic disruptor. The resulting suspension was centrifuged at $105000 \times g$ for 60 min and the precipitate was discarded.

Ammonium Sulfate Precipitation: A sufficient amount of 1 M potassium phosphate buffer (pH 7.2) was added to the supernatant solution to give a final concentration of 0.1 M potassium phosphate. Solid ammonium sulfate was added to give 80% saturation, and the resulting precipitate was collected and stored at −20°C.

Sephacryl S-200 Gel Filtration: The precipitate was redissolved in a minimum volume of 0.01 M potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA-2K, and dialyzed against the same buffer for 2 h. The dialysate was filtered through a Sephacryl S-200 Superfine column, eluting with 0.02 M potassium phosphate buffer (pH 7.0). The fractions containing high activity were combined, and after the addition of one-tenth volume of 1 M potassium phosphate buffer (pH 7.0), the protein was precipitated by adding ammonium sulfate to 80% saturation. The resulting precipitate was collected by centrifugation and stored at −20°C.

Diethylaminoethyl (DEAE)-Cellulose Column Chromatography: The protein was dissolved in a minimum volume of 0.01 M potassium phosphate buffer (pH 7.0) and passed through a column of Sephadex G-25 to remove the ammonium sulfate. The resulting solution was then adsorbed on a DEAE-cellulose column (10—15 mg protein per ml) which had been previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.0). The column was washed with the same buffer until no ultraviolet (UV)-absorbing compounds were eluted. At this point, the eluting buffer was changed to a mixture of 0.01 M potassium phosphate (pH 7.0) and 0.1 M potassium chloride. After all UV-absorbing materials had been eluted, the eluting buffer was changed to a mixture of 0.01 M potassium phosphate buffer (pH 7.0) and 0.2 M potassium chloride. The active fractions were combined, and the protein was precipitated at 80% saturation of ammonium sulfate after addition of one-tenth volume of 1 M potassium phosphate buffer (pH 7.0). The precipitated protein was collected as usual and stored at −20°C.

DEAE-Sepharose CL-6B Column Chromatography: The precipitate was dissolved in a minimum volume of 0.01 M potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and dialyzed against the same buffer for 2 h. The sample was applied to a DEAE-Sepharose CL-6B column equilibrated in the same buffer. The column was washed with the buffer until no UV-absorbing materials were eluted. The column was subsequently developed with a 0 to 0.4 M gradient of potassium chloride in the same buffer. The active fractions were combined, the protein was precipitated at 80% saturation of ammonium sulfate after addition of one-tenth volume of 1 M potassium phosphate buffer (pH 7.0). The precipitate was collected as usual and stored at −20°C.

Assay for trans-2-Enoyl-CoA Reductase——A typical assay mixture contained 20 nmol of substrate, 125 nmol of NADPH, 40 μmol of potassium phosphate (pH 6.5), and water to give a final volume of 0.80 ml. The reaction was initiated by adding a suitable amount of the enzyme preparation. One unit of the reductase was defined as the amount catalyzing the reduction of 1 μmol of substrate per min. In the experiments on heat denaturation, the protein precipitated after heating was removed by centrifugation, and the supernatant solution was used for the assay.

Fatty Acid Composition of Phospholipids——Total lipids were extracted from *E. coli* (0.9 g) by the procedure described by Folch *et al.*[11] Phospholipids were separated by silicic acid column (1.1 × 10 cm) chromatography, and then saponified by treatment with 15% methanolic potassium hydroxide at 70°C for 60 min. The resulting fatty acids were extracted in the usual way and methylated with diazomethane.

Gas Chromatography——Methyl esters of fatty acids were analyzed on a Shimadzu GC-4CM gas chromatography equipped with a hydrogen flame ionization detector. A glass column (3 mm × 2 m) of 10% diethyleneglycol succinate polyester (DEGS) on 60—80 mesh Shimalite W was used at 90°C for the analysis of medium chain acids and at 170°C for that of long chain acids.

Gas Chromatography-Mass Spectrometry (GC-MS)——A JEOL JMS-01SG-2 instrument coupled with a JEOL JMA-2000 mass data analysis system was employed. A glass column (2 mm × 1 m) of 10% DEGS on Shimalite W, 60—80 mesh, was used with helium as a carrier gas (0.4 kg/cm^2) and with an ionizing voltage of 25 eV. The column temperature was kept at 80°C for the analysis of medium chain acids and at 165°C for that of long chain acids.

Results and Discussion

Detection and Co-factor Requirements of the Reductase

Gel filtration of the crude extracts on Sephacryl S-200 Superfine led to the separation of the following four enoyl reductases with different co-factor requirements and substrate specificities. Maximal activities of NADH-dependent enoyl-ACP reductase (I) and NADPH-dependent enoyl-ACP reductase (II) were recovered in the same fraction (fraction number 52) as described by Weeks and Wakil.[5] An NADPH-dependent reductase (III) acting on *trans*-2-alkenoyl-CoA substrates, and an NADPH-dependent *cis*-2-enoyl-CoA reductase (IV)[2] were found in fractions 56 and 67, respectively.

The enoyl reductases I, II, and IV were eluted from a column of DEAE-cellulose with the buffer containing 0.1 M potassium chloride (or sodium chloride) as reported previously,[2,5] whereas the reductase III was recovered in the buffer containing 0.2 M potassium chloride. A preparation of the reductase III with a specific activity of 0.050 unit/mg obtained by DEAE-cellulose column chromatography was almost free of the reductases I, II, and IV.

These results indicate that the reductase III is distinct from the enoyl reductases which have previously been reported. Therefore, the reductase III is referred to as NADPH-dependent *trans*-2-enoyl-CoA reductase.

A preparation of the reductase with a specific activity of 0.079 unit/mg obtained by Sephacryl S-200 gel filtration was active on *trans*-2-hexenoyl-CoA and *trans*-2-octenoyl-CoA but was hardly active on *trans*-2-tetradecenoyl-CoA. The initial rates of reduction of *trans*-2-octenoyl-CoA were studied at various concentrations. The apparent Michaelis–Menten constant (K_m) for this substrate was calculated from double reciprocal replots of the same data to be 6.6×10^{-6} M.

The following experiments (optical pH determination, identification of the reaction product, heat stability measurements and inhibitor tests) were carried out using the preparation obtained by DEAE-cellulose column chromatography.

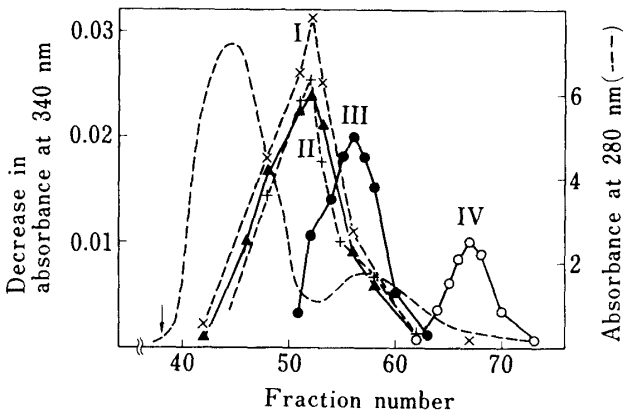


Fig. 1. Separation of Reductases by Gel Filtration of *E. coli* extracts on a Sephacryl S-200 Superfine Column

E. coli extracts (about 100 mg in 2 ml of 0.02 M potassium phosphate buffer, pH 7.0) were applied to a column (2.6×52 cm) of Sephacryl S-200 Superfine, and fractions of 3.0 ml were collected. Protein concentration was measured in terms of absorbance at 280nm (------). Reductase activities were measured as follows: NADH-dependent enoyl-ACP reductase (I), ×----× with *trans*-2-octenoyl-ACP and ▲——▲ with *trans*-2-octenoyl-CoA; NADPH-dependent enoyl-ACP reductase (II), +----+ with *trans*-2-octenoyl-ACP; NADPH-dependent *trans*-2-enoyl-CoA reductase (III), ●----● with *trans*-2-octenoyl-CoA; and NADPH-dependent *cis*-2-enoyl-CoA reductase, ○——○ with *cis*-2-octenoyl-CoA.

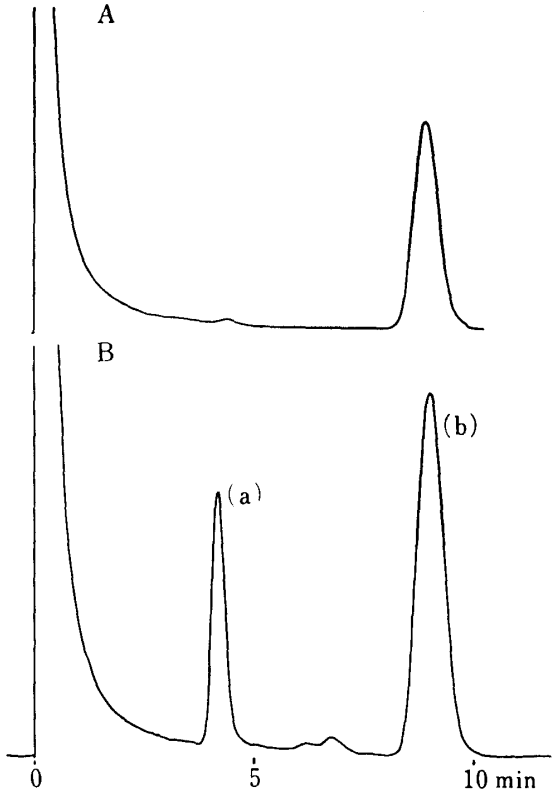


Fig. 2. Gas Chromatograms of Metabolite of *trans*-2-[6,7,8-$^2H_7$]-Octenoyl-CoA in the Presence of *trans*-2-Enoyl-CoA Reductase and NADPH

The complete reaction mixture contained 100 μmol of potassium phosphate (pH 7.0), 2 μmol of *trans*-2-[6,7,8-$^2H_7$]octenoyl-CoA, 2.5 μmol of NADPH, 0.1 unit of the reductase fraction obtained by gel filtration, and water to give a final volume of 1.0 ml. After an appropriate incubation time, the incubation mixture was treated as described previously.[1] Fatty acids extracted were methylated with diazomethane, and analyzed by gas chromatography. A, the complete system minus enzyme, minus NADPH, or minus substrate plus *trans*-2-[6,7,8-$^2H_7$]octenoic acid; B, incubation of the complete system for 120 min.

Optimal pH

When the rates of reduction of *trans*-2-octenoyl-CoA was measured in the range of pH from 5.0 to 9.0 (in 0.05 M potassium phosphate buffer, 0.05 M potassium phosphate dibasic-citrate buffer, and 0.05 M Tris-hydrochloride buffer), the enzyme preparation (DEAE-cellulose, 0.05 unit/mg) showed a broad pH optimun between 6 and 9.

Identification of the Reaction Product

The product of the reaction catalyzed by NADPH-dependent *trans*-2-enoyl-CoA reductase was examined by incubating the enzyme in a reaction mixture containing *trans*-2-[6,7,8-$^2H_7$]-octenoyl-CoA and NADPH. Aliquots of the reaction mixture were removed periodically and were saponified mildly,[1] then free fatty acids were extracted in the usual manner. The extracted acids were converted to their methyl esters by treatment with diazomethane, and aliquots were taken and analyzed by gas chromatography. One peak (a, in Fig. 2-B), which was not detected at zero time, increased with increasing incubation time. This peak was not detected in the control, as shown in Fig. 2-A. On the basis of its retention time, the peak was expected to be due to methyl [6,7,8-$^2H_7$]octanoate. Finally its structure was confirmed by GC–MS. The mass spectra of authentic [6,7,8-$^2H_7$]octanoate and the metabolite (peak a) were identical (Fig. 3).

Heat Stability

Even after leaving the reductase fraction (DEAE-cellulose, 1 mg/ml) at 0°C for 44 h at pH 7.0, 77% of its activity remained, indicating that the enzyme was rather stable. Then the effect of heat treatment on the reductase activity was tested. The crude enzyme preparation

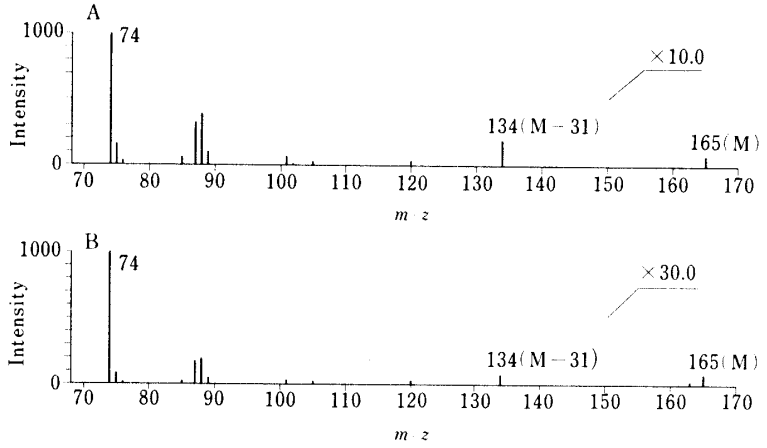


Fig. 3. GC–MS Analysis of the Metabolite Derived from [6,7,8-$^2H_7$]-Octenoyl–CoA

A: mass spectrum of authentic methyl [6,7,8-$^2H_7$]octanoate obtained by catalytic hydrogenation of *trans*-2-[6,7,8-$^2H_7$]octenoic acid over 5% palladium on barium sulfate, followed by its esterification with diazomethane.
B: mass spectrum of the metabolite (peak a, in Fig. 2-B).

Table I. Remaining Activities of NADPH-dependent *trans*-2-Enoyl-CoA Reductase after Heat Treatment

pH	65°C, 3 min	60°C, 8 min	56°C, 10 min	40°C, 15 min
6.0	11%	26%	50%	100%
7.0	15	33	90	100
8.0	30	52	100	100

An enzyme preparation (DEAE-cellulose, 0.05 unit/mg) was diluted to 4.26 mg protein concentration with 0.05 M potassiun phosphate (pH 6, 7, 8) and heat treatments were performed as indicated.

was subjected to different temperatures, and after removal of the denaturated protein by centrifugation, the supernatant fluid was subjected to assay. The reductase was found to be stable on heating at 40°C for 15 min, in the pH range 6—8. However, it was inactivated by 50% by treatment at 56°C for 10 min at pH 6, but retained its full activity at pH 8. It was considerably inactivated by heating at 60°C for 10 min (Table I).

Effect of Thiol Inhibitors

As shown in Table II, *p*-hydroxymercuribenzoic acid and *N*-ethylmaleimide were found to inhibit the reductase activity. *p*-Hydroxymercuribenzoic acid at a concentration of 1×10^{-6} M inhibited the reductase activity by 57%, while *N*-ethylmaleimide at a concentration of 1×10^{-3} M inhibited its activity by 37%. Iodoacetic acid was less effective, and even at a concentration of 1×10^{-3} M, no inhibition was observed. At a high concentration of 5×10^{-3} M, iodoacetic acid inhibited the activity by 60%. These observations suggest that this reductase has a functional thiol group which is sensitive to *p*-hydroxymercuribenzoic acid, but not to iodoacetic acid.

Cupric ion at a concentration of 5×10^{-5} M inhibited the reductase activity by 77%, and zinc ion at a concentration of 5×10^{-4} M inhibited the activity by 65% as shown in Table III. The other metal cations tested did not show any remarkable effect on the activity. No inhibition by cupric ion was observed in the presence of an excess of EDTA. It is known that cupric ion is a mercaptide-forming reagent with a small association constant.[12] These results also show that the enzyme has a functional thiol group.

Partial Purification of the Reductase

E. coli K-12 cells (13.4 g) grown in the medium supplemented with oleic acid were sonicated. After centrifugation, the supernatant fluid was treated at 56°C for 10 min, and

Table II. Effect of SH–Inhibitors on NADPH -dependent *trans*-2-Enoyl–CoA Reductase Activity

Inhibitor	Concentration (M)	Activity (Units)	Activity (%)
None		0.76	100
Iodoacetic acid	5×10^{-4}	0.78	103
	1×10^{-3}	0.71	93
	5×10^{-3}	0.30	40
N-Ethylmaleimide	1×10^{-4}	0.76	100
	5×10^{-4}	0.58	77
	1×10^{-3}	0.48	63
p-Hydroxymercuribenzoic acid	5×10^{-7}	0.76	100
	1×10^{-6}	0.33	43
	1×10^{-5}	0.30	40
	1×10^{-4}	0.28	37

Each inhibitor and 15 μg of the enzyme preparation (DEAE-cellulose, 0.05 unit/mg) were preincubated at 25°C for 10 min in 0.05 M potassium phosphate (pH 6.5), then 125 nmol of NADPH and 20 nmol of *trans*-2-octenoyl-CoA were added.

Table III. Effect of Metal Ions on NADPH-dependent *trans*-2-Enoyl–CoA Reductase Activity

Ions	Concentration (M)	Activity (Units)	Activity (%)
None		0.79	100
Cu^{2+}	1×10^{-5}	0.69	87
	5×10^{-5}	0.18	23
	5×10^{-4}	0.13	16
Zn^{2+}	1×10^{-4}	0.66	84
	5×10^{-4}	0.28	35
	1×10^{-3}	0.18	23
Fe^{3+}	5×10^{-4}	0.69	87
	1×10^{-3}	0.58	74
Mn^{2+}	1×10^{-3}	0.69	87
Mg^{2+}	1×10^{-3}	0.79	100
Ca^{2+}	1×10^{-3}	0.76	97
NH_4^+	1×10^{-3}	0.56	71
Na^+	1×10^{-3}	0.74	94

The reaction was initiated by the addition of 15 μg of the enzyme preparation (DEAE-cellulose, 0.05 unit/mg).

the resulting precipitate was removed by centrifugation. The protein in the supernatant was precipitated by the addition of ammonium sulfate. The precipitated protein was dissolved in the buffer, followed by dialysis. The fluid was then applied to a column (1.5 cm × 24 cm) of DEAE-Sepharose CL-6B and the enzyme was eluted with a 0 (120 ml) to 0.4 M (120 ml) gradient of potassium chloride, then precipitated by the addition of ammonium sulfate.

Table IV summarizes the results of the partial purification of the reductase. This preparation was free of the other reductases (I, II, and IV), although it was resolved into several components by polyacrylamide gel electrophoresis. The Km value for *trans*-2-octenoyl-CoA of this preparation was essentially the same as that obtained in the earlier experiment.

Induction of the Reductase

Crude extracts of *E. coli* cultured in a medium containing glucose or various fatty acids were passed through a Sephacryl S-200 column, and each fraction was assayed for the reductase.

A value for the specific activity of the reductase was determined in each case using the best fraction. The activity varied with the carbon source added to the culture medium. Table V shows that oleic acid and homo-oleic (*cis*-10-nonadecenoic) acid were the best carbon sources for inducing the reductase. The rate of induction was decreased by the addition of linoleic acid, elaidic acid, or glucose in that order.

TABLE IV. Partial Purification of *trans*-2-Enoyl-CoA Reductase from *E. coli*

Step	Total protein mg	Total activity		Specific activity	
		Units	(%)	Units/mg	(Fold)
Crude extracts	1160	38.2	(100)	0.033	(1.0)
Heat treatment	608	39.1	(102)	0.064	(2.0)
Ammonium sulfate	344	23.1	(60)	0.067	(2.0)
DEAE-Sepharose CL-6B	25.2	12.2	(32)	0.490	(14.7)

TABLE V. Activity of NADPH-dependent *trans*-2-Enoyl-CoA Reductase in Cell-free Extracts of *E. coli* Grown on Various Carbon Source Supplements

Carbon Source	Specific activity (units/mg)
Oleic acid	0.118
Homo-oleic acid	0.118
Linoleic acid	0.100
Elaidic acid	0.077
Glucose	0.045

The procedures for cultivation and assay were described under "Materials and Methods."

TABLE VI. Effect of Exogenous Fatty Acids on Fatty Acid Composition of Phospholipids of *E. coli*

	None (Glucose)	Oleic acid $C_{18:1}$	Linoleic acid $C_{18:2}$	Elaidic acid $C_{18:1}$ (*trans*)
C_{14}	2.6%	1.2%	1.4%	1.0%
C_{16}	45.8	38.1	39.8	24.6
C_{18}	0.4	<0.1	<0.1	
	48.8	39.4	41.3	25.6
$C_{14:1}$				1.5
$C_{16:1}$	7.1	12.4	4.3	17.4
$C_{18:1}$	7.5	32.6	3.7	28.2
	14.6	45.0	8.0	47.1
$C_{15}\triangle$				2.3
$C_{17}\triangle$	31.7	8.3	11.3	21.4
$C_{19}\triangle$	4.9	7.3	1.0	3.6
	36.6	15.6	12.3	27.3
$C_{16:2}$			33.0	
$C_{18:2}$			5.4	
			38.4	
Total	100.0	100.0	100.0	100.0

The number after C indicates the total number of carbon atoms and the number after the colon denotes the number of double bond(s), if any, present in the fatty acids. The symbol △ indicates the presence of a cyclopropane ring. The analytical procedures were described under "Materials and Methods."

It is not clear which is the actual inducer, the long chain unsaturated acids themselves or their metabolic intermediate(s) such as *cis*-3-alkenoyl-CoA and/or *cis*-2-alkenoyl-CoA. If the latter is the case, it is possible that both *cis*-3-alkenoyl-CoA and *cis*-2-alkenoyl-CoA act as inducers, since the reductase activity was induced both by the addition of oleic acid and by that of homo-oleic acid. However, linoleic acid showed less effect than oleic acid and homo-oleic acid, although its degradation produces both *cis*-3- and *cis*-2-intermediates.

If the former is the case, the melting point of the added unsaturated fatty acid may be an important factor in the induction. It was found that the lower the melting point of the fatty acid added, the better the induction of the reductase, with the exceptional case of linoleic acid. It has been reported that unsaturated fatty acids whose incorporation tends to increase the fluidity of membranes result in the induction of the chain elongation system in order to maintain the physical properties of the membranes under normal conditions in *Acholeplasma laidawii* B.[13] Also, in the case of *E. coli* cells, the supplemented unsaturated fatty acids were incorporated into phospholipids, and affected the fatty acid composition ratios (Table VI), suggesting that *trans*-2-enoyl-CoA reductase might be concerned in the homeostatic control of membrane functions.

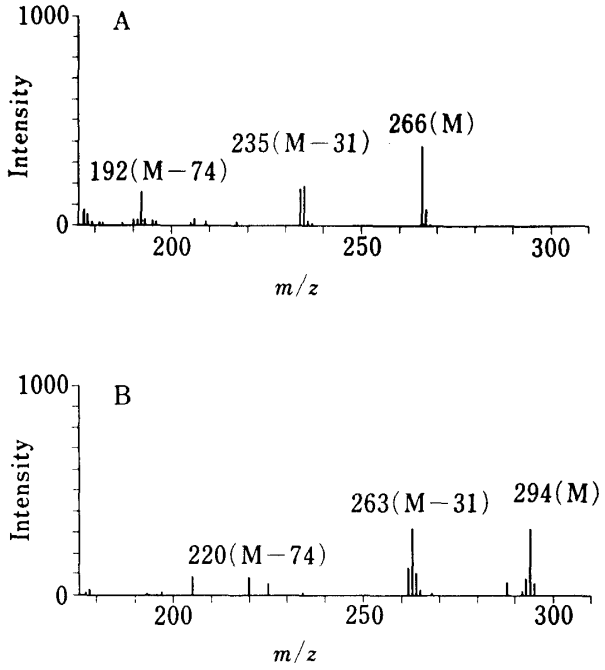


Fig. 4. GC–MS Analysis of Fatty Acid Components of Phospholipids of *E. coli* Grown in a Medium supplemented with Linoleic acid

A: mass spectrum of a metabolite (methyl ester).
B: mass spectrum of methyl linoleate.

On the other hand, when the cells were grown in the presence of unsaturated acids, there was an increase in the incorporation of the acid and its chain-shortened products. When the cells were cultured with oleic or eladic acid, considerable amounts of octadecenoic, hexadecenoic, and two cyclopropane acids were incorporated into their phospholipids. Analysis by GC–MS revealed that the cells grown with linoleic acid contained linoleic acid itself and its chain-shortened product, hexadecadienoic acid, neither of which are produced by *de novo* synthesis in *E. coli* cells (Fig. 4). It is considered that the chain-shortening system and the chain elongation system may act together to maintain membrane function constant.

trans-2-Enoyl–CoA reductase is reported to be a key enzyme for fatty acid chain elongation in rat liver,[14] and in *Mycobacterinm smegmatis*,[15] in which there is another chain elongation pathway specific for ACP esters.[16] The reductase may participate in the chain elongation system in *E. coli*. However, there has been no report on the existence of a CoA ester-dependent chain elongation system in *E. coli*, and no elongation of exogenous fatty acids was observed in *E. coli*.[17] The clarification of the physiological role of NADPH-dependent *trans*-2-enoyl-CoA reductase in *E. coli* requires further investigation.

References and Notes

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