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Studies on the Metabolism of Unsaturated Fatty Acids. V.[1] Isomerization of Thiol Esters of *cis*-2-Alkenoic Acids during Their Preparation and Alkaline Hydrolysis

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N-Acetylcysteamine and coenzyme A esters of *cis*-2-alkenoic acids have been found to undergo isomerization to the corresponding *trans*-isomers during their preparation by the mixed anhydride method and also during their alkaline hydrolysis. The isomerization might proceed by interaction of the free thiol group and the *cis*-double bond of 2-alkenoic thiol esters.

The use of pyridine as a base and three or more equivalents of the mixed anhydride to the thiol compound prevented the formation of the *trans*-isomer. Addition of hydrogen peroxide during alkaline hydrolysis also prevented the isomerization completely.

Keywords——*cis*-2-alkenoic acid; *cis*-2-octenoic acid; *trans*-2-octenoic acid; isomerization of *cis*-2-alkenoyl-CoA esters; isomerization of *cis*-2-alkenoyl-*N*-acetylcysteamine esters; mixed anhydride method; hydrogen peroxide

In our investigation of unsaturated fatty acid metabolism, we separated a new enzyme, *cis*-2-enoyl-coenzyme A (*cis*-2-enoyl-CoA) reductase from *Escherichia coli*,[2,3] and *Candida*.[4–6] It is considered that the reductase is responsible for the reduction of *cis*-2-alkenoyl-CoA intermediates formed during the β-oxidation of unsaturated fatty acids.

CoA esters of fatty acids have been prepared either by enzymatic[7,8] or by chemical methods.[9–15] The chemical syntheses are convenient for larger scale preparations. Among the various methods using acylating reagents such as acid chlorides, acid anhydrides, mixed anhydrides with ethyl chloroformate, *N*-hydroxysccinimide esters of fatty acids, and 1-acylimidazoles, the latter two are not suitable for the preparations of 2-alkenoyl-CoA esters.[14,15]

In preparations 2-alkenoyl-CoA esters by the mixed anhydride method, addition product smay be formed,[13] and especially in the case of *cis*-2-alkenoyl-CoA isomerization to the corresponding *trans*-isomer must be considered. However, there are no reports describing such non-enzymatic isomerization in detail, although Stoffel *et al.* prepared *cis*-2-enoyl-CoA by adding CoA to a solution containing a 30- to 40- fold excess of the mixed anhydride, presumably to prevent the isomerization.[16]

Contamination with the *trans*-isomer markes it difficult to assess the substrate specificity of *cis*-2-enoyl-CoA reductase, since there are reductases catalyzing the reduction of *trans*-2-alkenoyl-CoA esters in cell-free extracts of the microorganisms.[17] Furthermore, non-enzymatic isomerization of a *cis*-2-alkenoyl-CoA to the corresponding *trans*-2-alkenoic acid during usual alkaline hydrolysis has been observed by gas–liquid chromatography (GLC).

This paper deals with an improved method for the preparation of *cis*-2-alkenoyl-thiol esters and their hydrolysis in the presence of hydrogen peroxide to prevent isomerization of the *cis*-double bond.

Materials and Methods

Chemicals——CoA (2Na) and NADPH were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). *N*-Acetylcysteamine (NAC) was prepared according to the report of Kuhn and Quadbeck.[18] *cis*-2-Octenoic acid and *trans*-2-octenoic acid were synthesized according to the reports of Knight and Diamond[19] and

Boxer and Linstead,[20] respectively. These acids thus prepared were purified and characterized.[21]

Preparation of the Mixed Anhydride and Its Conversion to the Thiol Ester——A typical procedure is described below. *cis*-2-Octenoic acid (50 μmol) was dissolved in 1.6 ml of freshly distilled tetrahydrofuran (THF) and 50 μmol of pyridine was added to the solution. Then the solution was cooled to −10°C, 50 μmol of methyl chloroformate was added and the mixture was stirred at −10°C for 30 min. Then the mixture was cooled to −20°C and the precipitate formed was removed by filtration using a pipette fitted with a glass wool plug, and the filtrate was poured into a solution containing 16.7 μmol of NAC (or CoA) in 1.6 ml of 0.1 M potassium phosphate buffer (KPi) (pH 8.0). The mixture was stirred at 0°C for 15 min, then acidified with 3 N HCl, and THF was removed under reduced pressure. The residual solution was saturated with sodium chloride and extracted with ether. The extract was dried over anhydrous magnesium sulfate and the solvent was evaporated off. The residue was subjected to high performance liquid chromatography (HPLC).

In the case of CoA ester, the reaction mixture was lyophilized directly.

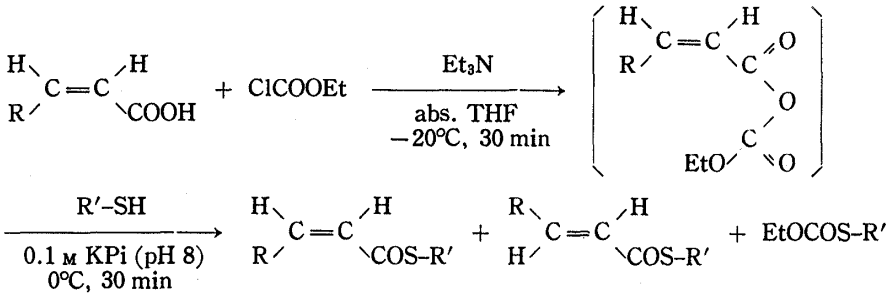

Chart 1

Alkaline Hydrolysis of Thiol Esters of *cis*-2-Alkenoic Acids in the Presence of Hydrogen Peroxide——A) Hydrolysis of NAC Ester: *cis*-2-Octenoyl-NAC (2.4 μmol) purified by HPLC was dissolved in 50% aqueous THF (0.4 ml), then 30% hydrogen peroxide solution (0.4 ml), 0.5 N NaOH solution (1 ml) and water were added to make a final volume of 2.0 ml. The mixture was allowed to stand at room temperature for 20 min, then acidified with 3 N HCl, and extracted with hexane. The extract was dried over anhydrous magnesium sulfate and the solvent was evaporated off under reduced pressure. The residue was treated with diazomethane and subjected to GLC.

B) Hydrolysis of CoA Esters: Crude *cis*-2-octenoyl-CoA (0.4 μmol) was suspended to 0.01 M KPi (pH 7.4, 30 μl) and applied to a thin-layer plate of cellulose (5 cm × 10 cm) in order to remove excess acid and unchanged CoA. The plate was developed at 5°C with butanol: glacial acetic acid: water (5: 2: 3).[22] After the development, the cellulose powder containing the thiol ester was scraped off and the thiol ester was eluted with 0.01 M KPi (pH 7.4, 1.1 ml) at 0°C. To the eluate, 30% hydrogen peroxide (0.4 ml) and 1 N NaOH solution (0.5 ml) were added, and the mixture was allowed to stand at room temperature for 20 min. The subsequent procedure was essentially the same as in the case of NAC ester described above.

GLC——Gas chromatographic data for NAC esters were obtained with a Shimadzu GC-4CM gas chromatograph equipped with a hydrogen flame ionization detector and a 2 m glass column packed with 3% OV-17. Operating conditions were as follows: the flow rate of carrier gas (N_2) was 60 ml/min; the temperature of the column oven was 240°C; the temperature of the injection and detector ports was 260°C. Fatty acid methyl esters were analyzed on a 2 m glass column packed with 10% DEGS at a column temperature of 90°C.

HPLC——A) NAC Ester: Experiments were carried out with a Hitachi 635 chromatograph equipped with a UV detector (263 nm), and a column (500 mm × 2.6 mm) packed with Hitachi silica gel # 3040. As the solvent, 0.4% v/v methanol in methylene chloride was used at a flow rate of 3 ml/min.

B) CoA Ester: The same instrument was used. Two 250 mm × 2.6 mm columns were packed with LiChrosorb-NH_2 and 0.02—0.1 M KPi (pH 6.5) was employed for linear gradient elution (2 ml/min).

High Resolution Mass Spectrometry——A JEOL JMS-01SG-2 instrument was operated at an ionizing voltage of 20 eV. The molecular ion of I was analyzed as follows:

Intensity	Observed	Error	Composition
78.8	243.1273	−2.0	$C_{12}H_{21}NO_2S....M^+$
28.0	244.1322	−4.8	$C_{12}H_{22}NO_2S....(M+1)^+$

Results and Discussion

NAC Esters of *cis*- and *trans*-2-Octenoic Acids

Since CoA is a relatively labile and complex molecule, NAC was employed as a model thiol compound. *cis*-2-Octenoic acid was employed as an acid because its CoA ester is a

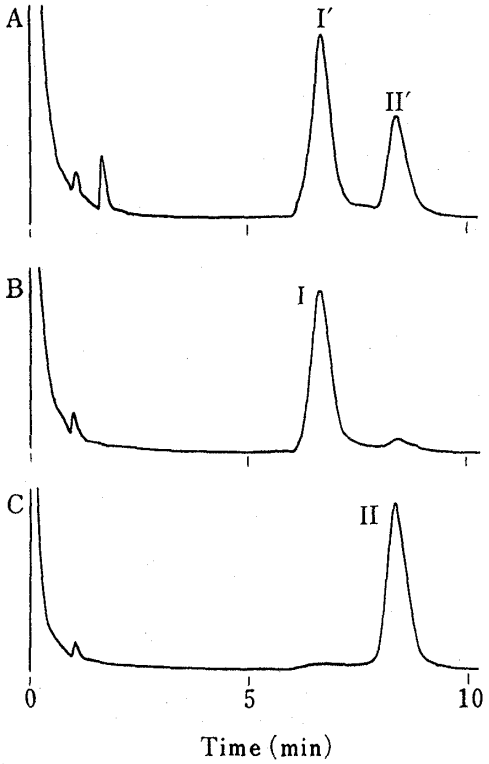

Fig. 1. Gas Chromatograms of 2-Octenoyl-NAC Derivatives

A: Reaction mixture for the preparation of *cis*-2-octenoic acid by the mixed anhydride method; B: one component (*cis*-2- octenoyl-NAC) separated by HPLC; C: the other component (*trans*-2-octenoyl-NAC) separated by HPLC. A Shimadzu GC-4CM gas chromatograph (FID) was employed with a glass column (2 m) packed with 3% OV-17. The column temperature was 240°C; the temperature of the injection port and detector was 260°C; the carrier gas (N_2) flow rate was 60 ml/min.

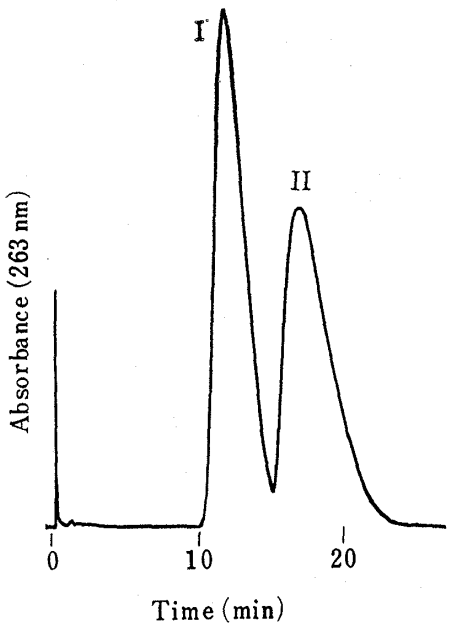

Fig. 2. Separation of 2-Octenoyl-NAC Derivatives by HPLC

A chromatogram obtained on a Hitachi 635 high performance liquid chromatograph with a UV detector (263 nm). A stainless steel column (500 mm × 2.6 mm) packed with Hitachi silica gel #3040 was used. Methanol in methylene chloride (0.4% v/v) was used as an eluting solvent at a flow rate of 3 ml/min.

representative intermediate containing a *cis*-2-double bond in the β-oxidation of unsaturated fatty acids. Synthesis of its thiol ester was tried firstly by the usual mixed anhydride method using triethylamine and ethyl chloroformate. When the reaction mixture was analyzed by GLC, there were two large peaks, designated I′ and II′ in the order of retention times (Fig. 1-A). Analysis of the reaction mixture by HPLC also gave two peaks, I and II, as shown in Fig. 2. Components I and II were separated by the same HPLC procedure by repeated injections of the mixture, and GLC analysis revealed that I was identical to I′ (Fig. 1-B), and II was identical to II′ (Fig. 1-C). Next, I was subjected to high resolution mass spectrometry. The molecular ion was observed and its elemental composition was determined as $C_{12}H_{22}NO_2S$, which was consistent with the molecular formula of octenoyl-NAC. Analysis of I by nuclear magnetic resonance (NMR) spectroscopy in the presence of a chemical shift reagent,[21] tris-(heptafluorobutanoylpivaloylmethanato)europium^{III} ($Eu(fod)_3$) indicated the presence of a *cis*-double bond at position 2 of its acyl moiety. The coupling constant of its double bond protons was determined to be 11.6 Hz by means of decoupling experiments.[22]

Based on these observations, the structure *cis*-2-octenoyl-NAC was assigned to I. It was also confirmed that II was *trans*-2-octenoyl-NAC by NMR spectroscopy (coupling constant: 16.0 Hz). The extent of isomerization was calculated to be about 40% from the areas of peaks I′ and II′ in Fig. 1-A.

Selective Preparation of *cis*-2-Octenoyl-NAC

To prevent the formation of *trans*-2-octenoyl-NAC, the effects of reaction temperature, acylating reagents, bases, and molar ratios of NAC with respect to the acid were investigated. Experiment 1 in Table I gave a mixture of *cis*-2-octenoyl-NAC (20%), *trans*-2-octenoyl-NAC (15%) and ethoxycarbonyl-NAC (12%), the structure of which was confirmed by NMR. The extent of isomerization was 42%, and did not vary with temperature between −20°C and −5°C, indicating that the isomerization is not temperature-dependent. When pyridine was employed instead of triethylamine, the formation of *trans*-2-octenoyl-NAC decreased considerably, while the yield of *cis*-2-octenoyl-NAC was almost constant and the formation of ethoxycarbonyl-NAC increased about 3—4 times. Therefore, several amines were employed and tested for effect on the isomerization (Table II). *N*,*N*-Dimethylaniline, the weakest base tested, did not give any reaction product. In the case of 1,8-diaza-bicyclo[5,4,0]undec-7-ene, the strongest base tested, it gave small amounts of *cis*-2-octenoyl-NAC and *trans*-2-octenoyl-NAC, and the extent of isomerization was more than 50%. The use of *N*-methylmorpholine gave better results than that of triethylamine, but considerable amounts of *trans*-2-octenoyl-NAC were formed in this case. The effects of acylating reagents are summarized in Table III. When pyridine was used as a base, the differences in the yields of *cis*-2-octenoyl NAC and in the isomerization ratios were small. On the other hand, when triethylamine was used, the extent of isomerization increased with increasing mass of the acyl group. Although the yield of *cis*-2-octenoyl-NAC increased to 32% with triethylamine and methyl chloroformate, the extent of isomerization remained at a high level. The molar ratios of NAC to *cis*-2-octenoic acid were changed as shown in Table IV. When the amount of NAC was limited to one-third or less, the formation of *trans*-2-octenoyl-NAC fell below 5%.

Table I. Effect of Reaction Temperature on the Preparation of *cis*-2-Octenoyl-NAC by the Mixed Anhydride Method

Expt. No.	1	2	3	4	5	6
Base	Et_3N	Et_3N	Et_3N	Pyridine	Pyridine	Pyridine
Temperature	−20°C	−10°C	−5°C	−20°C	−10°C	−5°C
Yield (%) *cis*[a]	20	32	20	23	22	20
trans[b]	15	20	13	3	2	2
EtOCOS–NAC	12	14	13	49	41	40
$\frac{[trans]\times 100}{[cis+trans]}$ (%)	43	38	39	12	8	9

a) *cis*-2-Octenoyl-NAC. *b*) *trans*-2-Octenoyl-NAC.
Each flask contained one equivalent of Et_3N (Experiments 1—3) or pyridine (Experiments 4—6) and the temperature was changed as indicated. Other components and conditions were the same as described in "Materials and Methods."

Table II. Effect of Bases on the Preparation of *cis*-2-Octenoyl-NAC by the Mixed Anhydride Method

Expt. No.	1	2	3	4
Base	Pyridine	MM[a]	Et_3N	DBU[b]
Yield (%) *cis*	24	16	22	4
trans	2	10	19	6
EtOCOS–NAC	46	10	8	27
$\frac{[trans]\times 100}{[cis+trans]}$ (%)	8	38	46	60

a) *N*-Methylmorpholine.
b) 1,8-Diaza-bicyclo[5,4,0]undec-7-ene.

Table III. Effect of Acylating Reagents on the Preparation of *cis*-2-Octenoyl-NAC by the Mixed Anhydride Method

Expt. No.	1	2	3	4	5	6
Base	Et_3N	Et_3N	Et_3N	Pyridine	Pyridine	Pyridine
ClCOOR (R=)	Me	Et	iso-Bu	Me	Et	iso-Bu
Yield (%) *cis*	32	26	12	22	22	20
trans	25	28	20	1	2	1
ROCOS–NAC	12	7	8	22	46	62
$\frac{[trans]\times 100}{[cis+trans]}$ (%)	44	52	63	4	8	5

Table IV. Effect of NAC–SH Concentration on the Preparation of *cis*-2-Octenoyl-NAC by the Mixed Anhydride Method

Expt. No.	1	2	3	4	5
$\frac{[NAC-SH]}{[Free\ acid]}$	2	1	1/3	1/5	1/8
Yield (%) *cis*	1	10	18	21	17
trans	7	10	1	1	1
EtOCOS–NAC	37	24	31	49	54
$\frac{[trans]\times 100}{[cis+trans]}$ (%)	88	50	5	5	6

Isomerization of *cis*-2-Octenoyl-NAC to the *trans*-Isomer

The synthesis of *cis*-2-alkenoyl-NAC consists of two reactions: the formation of the mixed anhydride and the transfer of the acyl group to NAC. In order to clarify the reaction responsible for the isomerization, several sets of experiments were carried out. When free *cis*-2-octenoic acid was treated with triethylamine, ethyl chloroformate, and NAC, no *trans*-2-octenoic acid was detected in the reaction mixture, indicating that the isomerization did not proceed during the formation of the mixed anhydride. On the other hand, when *cis*-2-octenoyl-NAC isolated by HPLC was allowed to stand in the presence of free NAC at 0°C for 30 min, more than 60% of the material was converted to *trans*-2-octenoyl-NAC, regardless of the presence or absence of a base (Table V).

Table V. Isomerization of *cis*-2-Octenoyl-NAC to the *trans*-Isomer

$$\text{RCOS–NAC} \xrightarrow[\text{THF+0.1M KPi (pH 8)}\ 0°C,\ 30\ min]{\text{base, NAC–SH}} \text{isomerization}$$

Expt. No.	Base	NAC–SH	$\frac{[trans]\times 100}{[cis+trans]}$ (%)
1	Et_3N	−	8
2	—	+	66
3	Et_3N	+	69
4	Pyridine	+	66

On the basis of these results, we concluded that the isomerization did not occur in the free acid, but in the thiol ester, in which the *cis*-2-double bond of the acyl group is activated to react with the free thiol group of unchanged NAC to form a Michael-addition product-like intermediate. As mentioned above, the fact that little isomerization proceeded when pyridine

was employed as a base instead of triethylamine may be explained as follows: the use of pyridine caused an increase in the amount of ethoxycarbonyl-NAC, which decreased the concentration of unchanged free NAC in the reaction mixture, and thus tended to prevent the isomerization. In general, the use of triethylamine results in good yields in the preparation of thiol esters of fatty acids other than *cis*-2-alkenoic acids.

Preparation of *cis*-2-Octenoyl-CoA

On the basis of the results obtained in the model experiments, the CoA ester of *cis*-2-octenoic acid was prepared. The reaction mixture consisted of pyridine, methyl chloroformate, and 3 mol of *cis*-2-octenoic acid to 1 mol of CoA. Since free CoA, *cis*-2-octenoyl-CoA, and its *trans*-isomer were separated by HPLC with a LiChrosorb-NH_2 column as shown in Fig. 3-A, the resulting products were analyzed under the same conditions. The peak of the *trans*-isomer in the products was barely observed at the position indicated by the arrow (Fig. 3-B). Since it is considered that the sensitivity of a UV detector for both isomers is equal, the extent of the isomerization of *cis*-2-octenoyl-CoA during the procedure was negligible.

Hydrolysis of CoA Ester in the Presence of Hydrogen Peroxide

During the hydrolysis of *cis*-2-octenoyl-NAC (or *cis*-2-octenoyl-CoA) in the absence of hydrogen peroxide, a considerable amount of the *cis* double bond was converted to the *trans*

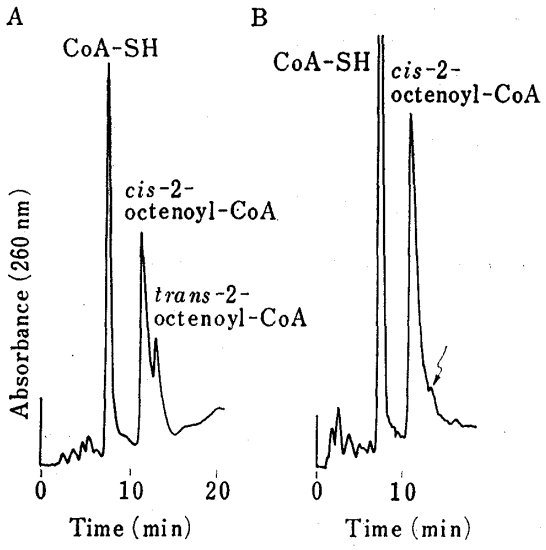

Fig. 3. Separation of 2-Octenoyl-CoA Derivatives by HPLC

A: a chromatogram of a mixture containing CoA, and *cis*-and *trans*-2-octenoyl-CoA esters. B: a chromatogram of a *cis*-2-octenoyl-CoA preparation obtained by the mixed anhydride method using pyridine as a base. A Hitachi 635 high performance liquid chromatograph with a UV detector (260 nm) was employed. Two stainless steel columns (250 mm × 2.6 mm each) packed with LiChrosorb-NH_2 were used, and 0.02—0.1 M KPi (linear gradient, pH 6.5) was used as an eluting solvent at a flow rate of 2 ml/min.

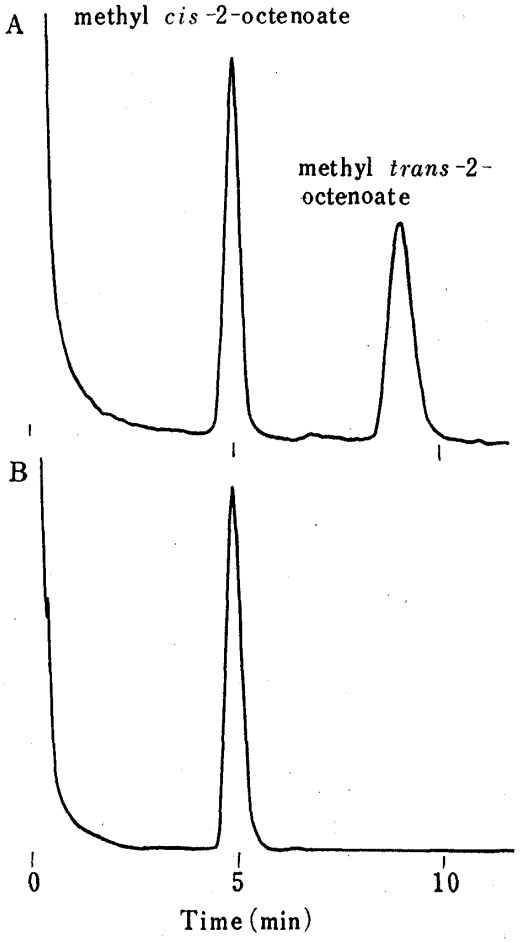

Fig. 4. Isomerization during Alkaline Hydrolysis of *cis*-2-Octenoyl-NAC

Gas chromatograms of the hydrolysate (Me ester) treated in the absence (A) or in the presence (B) of hydrogen peroxide are shown. A 2 m glass column packed with 10% DEGS was used at a column temperature of 90°C.

configuration, as shown in Fig. 4-A. On the other hand, in the presence of hydrogen peroxide at a final concentration of 6%, no isomerization was observed (Fig. 4-B). It is presumed that hydrogen peroxide oxidized the thiol group of NAC, which was liberated during the hydrolysis, to the disulfide. A free thiol group is required for the isomerization. It is possible to estimate the true isomerization rate in a preparation of *cis*-2-alkenoyl-CoA by analyzing the composition of liberated fatty acid without isomerization during alkaline hydrolysis in the presence of hydrogen peroxide.

Enzymatic Determination of *cis*-2-Octenoyl-CoA

The purification of the CoA ester was performed by chromatography on a cellulose column or plate.[22] When an aliquot of separated *cis*-2-octenoyl-CoA fraction was incubated in the presence of NADPH-dependent *cis*-2-enoyl-CoA reductase which had been isolated from *E. coli*,[2,3] consumption of NADPH was observed spectrophotometrically. However, when an aliquot of the fraction was incubated in the presence of NADPH-dependent *trans*-2-enoyl-CoA reductase which had also been isolated from *E. coli*,[17] no consumption of NADPH was recorded, indicating that the preparations of the *cis*-2-alkenoyl-CoA had been successful (Fig. 5).

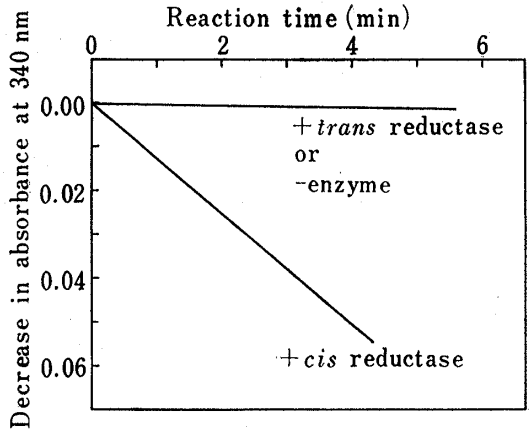

Fig. 5. Enzymatic Determination of *cis*-2-Octenoyl-CoA

The complete reaction mixture contained 20 nmol of *cis*-2-octenoyl-CoA, 125 nmol of NADPH, 40 μmol of KPi (pH 7.2), and the enzyme fraction in a final volume of 0.80 ml.

It is said that *cis*-2-alkenoyl-CoA intermediates have not been detected as metabolites of fatty acid degradation.[23] Although various explanations have been proposed for this phenomenon, it is possible that a physiological amount of *cis*-2-alkenoyl-CoA esters is converted to the corresponding *trans*-isomers during hydrolysis of their CoA esters by the mechanism mentioned above. Addition of hydrogen peroxide to a solution containing metabolites of fatty acid degradation should make it possible to obtain reliable information.

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