Specificity of Cyclopropane Fatty Acid Synthesis in *Escherichia coli*. Utilization of Isomers of Monounsaturated Fatty Acids†

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ABSTRACT: The conversion of a series of monounsaturated fatty acids to cyclopropane acids has been investigated in *Escherichia coli* K1060, an unsaturated fatty acid auxotroph which is also blocked in fatty acid degradation. In overnight cultures almost complete conversion of *cis*-9-hexadecenoic acid was observed. Substantial conversion of *cis*-10-hexadecenoic acid, of *cis*-9- and of *cis*-11-octadecenoic acids was also seen, ranging from 29 to 38% of the incorporated unsaturated acids. Eleven per cent conversion of incorporated *cis*-11-hexadecenoic acid to a cyclopropane acid was observed under similar conditions. Cyclopropane acids formed from all of these precursors had retention times on an open capillary gas–liquid chromatography column consistent with addition of the methylene bridge at the position of the double bond. There was little or no detectable conversion of *cis*-6-hexadecenoic, *cis*-7-hexadecenoic, and *cis*-6-octadecenoic acids to cyclopropane fatty acids. The positions of four of the hexadecenoic acid isomers, cis-7, cis-9, cis-10, and cis-11, on phosphatidylethanolamine were determined by snake venom treatment and all were found mainly on carbon-2 of the *sn*-glycerol 3-phosphate backbone. All the fatty acids tested were capable of supporting as rapid growth of the auxotroph as the natural isomers palmitoleic acid (*cis*-9-hexadecenoic acid) and *cis*-vaccenic acid (*cis*-11-octadecenoic acid).

Cyclopropane fatty acids are found in a wide variety of Gram-positive and Gram-negative bacteria (Goldfine, 1972). They have also been found in certain protozoa (Meyer and Holz, 1966), millepedes (Oudejans *et al.*, 1971) and in the rumen tissues of sheep (Body, 1972). In bacteria they are formed by the transfer of the methyl group of *S*-adenosylmethionine to the double bond of an unsaturated fatty acid present in a phospholipid (Law, 1971). Cyclopropane fatty aldehydes and alcohols can be formed by a similar addition of a C_1 unit to an unsaturated alk-1-enyl ether containing phospholipid (Chung and Goldfine, 1965) or an alkyl ether phospholipid (Thomas and Law, 1966).

Thomas and Law (1966) have studied the specificity of the reaction for unsaturated fatty acids in phospholipids with different polar groups and have also demonstrated an absolute specificity for the *sn*-glycerol 3-phosphatides. Although the enzyme(s) from *Clostridium butyricum* can transfer a C_1 unit to either the 1-acyl or 2-acyl group on a phospholipid, it shows some preference for the 1-acyl group (Law, 1971). The commonly occurring bacterial cyclopropane fatty acids are *cis*-9,10-methylenehexadecanoic acid, which is derived from palmitoleic acid (Kaneshiro and Marr, 1961), and *cis*-11,12-methyleneoctadecanoic acid, lactobacillic acid, which is derived from *cis*-vaccenic acid (Hofmann and Lucas, 1950; Hofmann, 1963). *cis*-9,10-Methyleneoctadecanoic acid (dihydrosterculic acid) has also been found in bacteria (Gray, 1962; Goldfine and Panos, 1971). A study of the cyclopropane fatty acids and aldehydes of *Clostridium butyricum* revealed a strong apparent specificity of the cyclopropane synthetase for *cis*-9,10-hexadecenoic acid, palmitoleic acid, rather than the cis-7,8 isomer, which is also present in the phospholipids of this organism. An examination of the C_{19} cyclopropane fatty acids revealed some preference for formation of the cis-11,12 isomer over the cis-9,10 isomer, even though there were approximately equal amounts of the two precursors in the cellular phospholipids (Goldfine and Panos, 1971).

In the present work we have studied the formation of cyclopropane fatty acids in *Escherichia coli* from hexadecenoic acid isomers ranging from cis-6 to cis-11 and from three octadecenoic acids. We have utilized an unsaturated fatty acid auxotroph, which is also blocked in the oxidation of fatty acids, in order to obtain cells with phospholipids containing the fatty acid isomer that was incorporated into the growth medium.

Materials

Lithium amide was purchased from Matheson, Coleman & Bell; 6-bromohexanoic acid from Fisher Scientific Co.; 1-decyne from Chemical Procurement Labs, College Point, N. Y.; 10-bromodecanoic acid was purchased from K & K Laboratories, Plainview, N. Y.; 1-hexyne from Farchan Research Laboratories, Willoughby, Ohio; and 5% palladium on barium sulfate from Engelhard Industries. Palmitoleic acid and NIH-D standard fatty acid mixture were products of Applied Science Laboratories Inc., State College, Pa. Palmitelaidic acid was obtained from Nu-chek Prep, Elysian, Minn. *cis*-9,10-Methylenehexadecanoic acid was purchased from Analabs, Inc., North Haven, Conn. Dr. K. Hofmann kindly provided *cis*-11,12-methyleneoctadecanoic acid. Dried venom of *Ancistrodon piscivorus piscivorus* was purchased from Sigma Chemical Co., St. Louis, Mo. 10-Hexadecynoic acid was the generous gift of F. Gunstone. Glass-distilled solvents were purchased from The Anspec Co., Ann Arbor, Mich. *Thunbergia alata* seeds were the generous gift of G. F. Spencer. *E. coli* strain K 1060, an unsaturated fatty acid auxotroph blocked in β oxidation, originally isolated in P. Overath's Laboratory, was obtained from D. F. Silbert.

Methods

Synthesis of long-chain acetylenic acids (7-hexadecynoic and 11-hexadecynoic acids) was achieved by employing a

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modification of the procedure of Ames and Covell (1963). A solution of 1-alkyne (0.24 mol) in dry tetrahydrofuran (20 ml) was added dropwise over 1 hr to a warm (50–60°) stirred suspension of lithium amide (0.24 mol) in dry tetrahydrofuran (150 ml). After addition was completed, the mixture was refluxed under N_2 for 1 hr. To the reaction mixture was then added a solution of the ω-bromo acid (0.024 mol) in dry tetrahydrofuran (20 ml) dropwise over a 5–10-min period. The reaction mixture was refluxed an additional 8 hr. Tetrahydrofuran was then evaporated on a rotary evaporator and the suspension was acidified with 5 N HCl followed by extraction three times with diethyl ether. The combined ether extracts were made alkaline with 10% NaOH and extracted three times with diethyl ether in order to remove unreacted 1-alkyne. The aqueous phase was reacidified with 5 N HCl and extracted three times with diethyl ether. The combined ether extracts were dried over Na_2SO_4 overnight. In the case of 7-hexadecynoic acid, the washed and dried ether extracts were evaporated and the resultant product was distilled under reduced pressure. The product was then analyzed by nuclear magnetic resonance and infrared spectroscopy; both methods gave the expected spectra. *cis*-7-Hexadecenoic acid and *cis*-10-hexadecenoic acid were made by subjecting the corresponding acetylenic acids to partial hydrogenation at atmospheric pressure as described by Ames and Covell (1963). Products of hydrogenation were filtered, dissolved in diethyl ether, and acidified with 1 N HCl. The organic phase was washed three times then dried over Na_2SO_4 and evaporated to recover the monoenoic fatty acid products. Crude 11-hexadecynoic acid was also partially hydrogenated at atmospheric pressure, to yield *cis*-11-hexadecenoic acid. The product was methylated with diazomethane and purified by preparative thin-layer chromatography on 500-μ, 20% $AgNO_3$-impregnated silica gel G plates with a developing solvent system of hexane–chloroform–methanol (30:68:2, v/v). The resultant bands were visualized under ultraviolet light after spraying the plates with an ethanolic solution of 0.05% Rhodamine 6G, and the purified product was eluted from the silica gel with diethyl ether.

cis-6-Hexadecenoic acid is the major monoenoic component of *Thunbergia alata* seed oil (Spencer *et al.*, 1971) which was extracted from ground seeds with petroleum ether (bp 30–60°). After saponification, *cis*-6-hexadecenoate was isolated by preparative gas–liquid chromatography of methylated fatty acids on a column (0.5 in. × 12 ft) of 10% butanediol succinate on $Me_3SiOSiMe_3$-treated Chromosorb W (80–100 mesh) at 190°. A sample of each fatty acid synthesized was methylated with diazomethane and analyzed by gas–liquid chromatography on a column ($^1/_8$ in. × 6 ft) of 10% EGSS-X on Gas Chrom P (100–120 mesh) at 180° in order to determine the purity of the compound. They were also chromatographed on a 0.01 in. × 150 ft open tubular capillary column (see below) in order to determine the purity of the positional isomer.

E. coli K1060 F$^-$thi$^-$lac^c*fab*B, *fad* E, was grown at 30° on a New Brunswick reciprocating shaker on medium E (Vogel and Bonner, 1956) containing 0.4% glycerol as carbon source, 3×10^{-6} M thiamine, 0.0005% yeast extract (Silbert *et al.*, 1968), and supplemented with 0.04% Brij 35 (polyoxyethylene-23-lauryl ether) and 0.01% potassium salt of the appropriate unsaturated fatty acid transferred from ethanolic 5% stock solutions. After overnight growth in 2.0 ml of oleate-supplemented medium, cultures were diluted 10- to 100-fold for overnight growth in the appropriate hexadecenoate or octadecenoate-supplemented medium. These cultures were then diluted 5- to 10-fold to give a final volume of 50 ml of the same medium and again grown overnight. In a few cases, when more cells were desired, a third subculture of 200 ml of the same medium was started by a 10-fold dilution and grown overnight. Cells were harvested by centrifugation at 6000*g* for 15 min and were washed once with 50 mM phosphate buffer (pH 7.2). Cellular lipids were extracted by the procedure of Bligh and Dyer (1959) and subjected to alkaline hydrolysis (Rooney *et al.*, 1972). Fatty acids were extracted, methylated with diazomethane, and analyzed by gas–liquid chromatography. Quantitative analysis for the measurement of the relative amounts of hexadecenoic and cyclopropane fatty acids was carried out by gas–liquid chromatography on a 10% EGSS-X on Gas Chrom P column as described above. The Perkin-Elmer Model 990 was equipped with a hydrogen flame detector and in the later parts of this work with a Model CR3-208 Infotronics digital electronic integrator. The accuracy of the instrument was checked with an NIH-D standard mixture of similar chain length saturated and monounsaturated fatty acids. Analysis for positional isomers of the monoenoic and cyclopropane fatty acids was carried out on a polar capillary column (0.01 in. × 150 ft) coated with Carbowax K20-M plus V-930 (99:1) as has been described (Panos, 1965; Panos and Henrikson, 1968). The column temperature was 186° and the detector was 230°.

The phosphatidylethanolamine fraction from the total lipids of each sample was isolated on silica gel G thin-layer plates with the solvent system $CHCl_3$–MeOH–7 N NH_4OH (65:35:5, v/v) (Baumann *et al.*, 1965) and eluted with $CHCl_3$–MeOH–H_2O–formic acid (97:97:4:2, v/v). Two mg of *Ancistrodon piscivorus piscivorus* venom in 0.25 ml of 0.1 M Tris-HCl buffer (pH 7.2) and 0.25 ml of 0.005 M $CaCl_2$ was added to the phosphatidylethanolamine (1.2–2.6 mg) dissolved in 2.0 ml of glass-distilled diethyl ether. The reaction mixture at room temperature was shaken by hand at 10–15-min intervals or continuously on a mechanical shaker for a total of 18–21 hr. The reaction was terminated by addition of 2.5 ml of 95% ethanol and samples were evaporated to dryness with a stream of nitrogen gas, redissolved, and chromatographed on silica gel G plates developed with $CHCl_3$–MeOH–7 N NH_4OH (65:35:5, v/v). Lipids were visualized by spraying with water, dried, scraped, and eluted with $CHCl_3$–MeOH (1:2, v/v). Exposure of a peripheral region of each sample left on the plates to iodine vapor, and following evaporation of I_2, treatment with ninhydrin reagent, confirmed the absence of PE,[1] indicating complete hydrolysis. Fatty acids cleaved from the C-2 position of PE by snake venom phospholipase A_2, along with C-1 position fatty acids, obtained by alkaline hydrolysis of lyso-PE, were methylated and analyzed by gas–liquid chromatography on a packed column as described above. In the above preparative procedures, thin-layer plates were always prerun in the same solvent system before the samples were applied.

Results

Gas–Liquid Chromatography of Hexadecenoic Acid Isomers. The behavior of the methyl esters of the synthetic and natural hexadecenoic acid isomers on an open tubular capillary column of Carbowax K20-M plus V-930 (99:1) is given in

[1] Abbreviation used is: PE, phosphatidylethanolamine. For monounsaturated acids, the number before the colon is the chain length. The position of the double bond is indicated as cis-9, etc; cyc, cyclopropane acid, *e.g.*, 17:cyc-9,10. The last two numbers give the ring position.

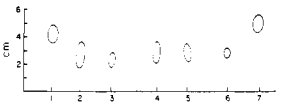

FIGURE 1: Separation of cis and trans isomers by thin-layer chromatography on 10% $AgNO_3$-impregnated silica gel G in a solvent system of petroleum ether–ether (95:5, v/v): 1,trans-9-16:1; 2, cis-6-16:1; 3, cis-7-16:1; 4, cis-9-16:1; 5, cis-10-16:1; 6, cis-11-16:1; 7, trans-9-18:1. The solvent front was approximately 19 cm from the origin.

Table I. As expected from results obtained with *cis*-octadecenoic acid isomers (Ackman, 1972), the cis-6 and cis-7 isomers were not resolved. The other isomers were resolved from each other and from the cis-6 and cis-7 isomers. All the synthesized isomers appeared to be free of other isomers as judged by capillary column gas–liquid chromatography. The cis-6 isomer isolated from *Thunbergia alata* seed oil would be expected to have a 2.0% contamination with the cis-7 isomer (Spencer *et al.*, 1971). That all of the isomers were indeed cis was demonstrated by argentation thin-layer chromatography (Figure 1). Gas chromatography revealed 1.1% contamination of both the cis-7 and cis-10 isomers with 16:0 resulting from slight excess reduction of the hexadecynoic acids. Cis-6-16:1 had 4.0% contamination with 16:0. Since the saturated compound is made in abundance by *E. coli*, we did not attempt to remove the 16:0 contaminant prior to the feeding experiments.

Conversion of Hexadecenoic Acid Isomers to Cyclopropane Acids. All of the hexadecenoic acids tested were capable of supporting the growth of the unsaturated fatty acid auxotroph (Figure 2). They gave similar growth yields as measured by either final turbidity or wet weight of cells with the possible exception of cis-6-16:1, which produced a final turbidity one-third less than the other supplements in one experiment, but the same wet weight of cells as the others in a separate experiment. In order to test for conversion of these isomers to the corresponding cyclopropane fatty acids, the cells were allowed to grow overnight into the stationary phase before they were harvested by centrifugation. The fatty acids obtained by saponification of the chloroform–methanol-extractable lipids were converted to the methyl esters and separated on a packed column by gas–liquid chromatography. The amounts of hexadecenoic acid and C_{17}-cyclopropane acid (17:cyc) were determined by measuring the peak areas by triangulation (Table II).

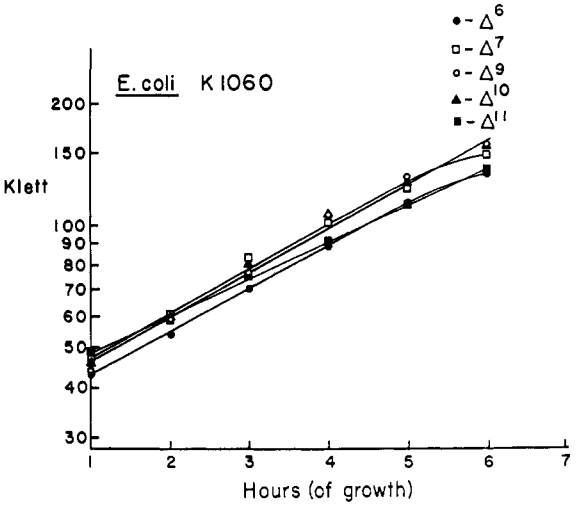

FIGURE 2: Growth of *E. coli* K1060 on isomeric hexadecenoic acids.

In order to obtain information on the location of the cyclopropane rings, capillary column gas chromatography of the methyl esters isolated from the total extractable cellular lipids was carried out. The retention times relative to 16:0 methyl ester of the C_{17}-cyclopropane fatty acid methyl esters are given in Table III. The small amounts of cyclopropane fatty acids formed in the cells grown on cis-6 and cis-7-16:1 had the same retention time as *cis*-9,10-methylenehexadecanoate. Given the power of the column to resolve positional isomers of cyclopropane fatty acids (Panos and Henrikson, 1968), and the absence of double-bond isomerization before alkylenation (McCloskey and Law, 1967), the cyclopropane fatty acids were not formed from the cis-6 and cis-7 isomers fed to the cells, but were probably formed from small amounts of cis-9-16:1 present in the cells. In the case of cells grown on cis-10-16:1, the cyclopropane fatty acid formed had a retention time consistent with *cis*-10,11-methylenehexadecanoate. The gas chromatographic separation factor (Ackman, 1969) for the cyclopropane fatty acid found in these cells from the corresponding unsaturated fatty acid methyl ester (1.44) is

TABLE I: Gas Chromatographic Behavior of Methyl *cis*-Hexadecenoates.[a]

Isomer	Retention Rel to 16:0 Methyl Ester	ECL[b]
Cis-6-16:1	1.083	16.22
Cis-7-16:1	1.080	16.21
Cis-9-16:1	1.103	16.27
Cis-10-16:1	1.124	16.32
Cis-11-16:1	1.155	16.39
Cis-11-16:1[c]	1.153	

[a] Chromatographed on Carbowax K20-M plus V-930 (99:1). For conditions, see Methods. Retention times corrected for column dead volume by observation of a solvent peak. [b] ECL = equivalent chain length determined graphically from retention of 14:0 and 16:0 methyl esters (Ackman, 1969). Relative retention of 14:0 was 0.486. [c] Isolated from *Cytophaga hutchinsonii* lipids (Walker, 1969). Kindly provided by Dr. Walker.

TABLE II: Conversion of Hexadecenoic Acid Isomers to Cyclopropane Fatty Acids.

Isomer Fed to Cells	Cellular Fatty Acids: 16:1 (Wt % of Total Fatty Acids)	17:cyc	17:cyc Cor	[17:cyc/(16:1 + 17:cyc)] × 100 (%)
Cis-6-16:1	33.3	1.06	nd[a]	<0.5
Cis-7-16:1	41.5	1.18	nd[a]	<0.5
Cis-9-16:1	3.57, 6.92[c]	47.5, 27.1		93, 73.8
Cis-10-16:1	26.7	16.1		37.6
Cis-11-16:1	35.9	9.1	4.5[b]	11.1[d]

[a] nd, no detectable 17:cyc other than 17:cyc-9,10. [b] Corrected for presence of 17:cyc-9,10 measured after capillary column chromatography. [c] All values represent the average of duplicate determinations on lipids from single batches of cells. Data for cis-9-16:1 represent two batches of cells. [d] Using the 17:cyc (cor) value from column 3.

TABLE III: Gas Chromatographic Behavior of 17-Carbon Cyclopropane Acid Methyl Esters.

16:1 Isomer Added to Medium	Rel Retention of 17:cyc[a]
Cis-6	1.591
Cis-7	1.590
Cis-9	1.592
Cis-10	1.615
Cis-11	1.593, 1.648
Standard cis-9,10-17:cyc	1.591

[a] Retention relative to that of 16:0 methyl ester; average of 2–4 runs. Retention times corrected for column dead volume by observation of solvent peak.

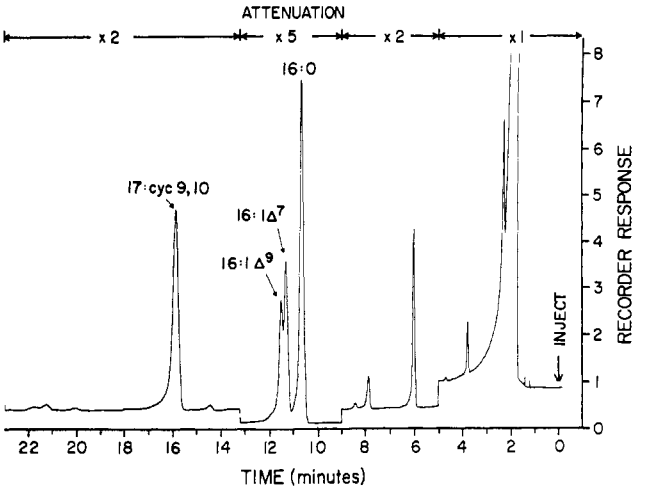

FIGURE 3: Capillary column gas–liquid chromatography of the methyl esters of fatty acids isolated from *E. coli* K1060 lipids when the cells were grown on a mixture of cis-7-16:1 and cis-9-16:1.

identical with the separation factor for *cis*-9,10-methylenehexadecanoate and cis-9-16:1 (1.44). In the cells grown on cis-11-16:1, two C_{17}-cyclopropane fatty acids were detected by capillary gas chromatography. One, representing 51% of the total C_{17}, and 4.6% of the total fatty acids, had the same retention time as *cis*-9,10-methylenehexadecanoate. The other C_{17} fatty acid had the retention time expected for *cis*-11,12-methylenehexadecanoate. The separation factor from the corresponding unsaturated fatty acid, cis-11-16:1, was 1.43. Thus the amount of cyclopropane fatty acid actually formed from cis-11-16:1 is 49% of the total C_{17}-cyclopropane fatty acids measured on the packed column. The value given in Table II was corrected accordingly (column 3). The small amounts of *cis*-9,10-methylenehexadecanoate found in cells grown on cis-6, cis-7, and cis-11-16:1 were presumably formed from endogenous cis-9-16:1, which may have come from variable numbers of revertants present in the mutant population.

It is clear that under conditions in which cis-9-16:1 was converted 73.8 and 93% to the corresponding cyclopropane fatty acid in two experiments, there was no detectable methylenation of cis-6 or cis-7-16:1. Only 11% of cis-11-16:1 was converted to the cyclopropane compound and the cis-10-16:1 isomer was methylenated 37.6% in overnight culture. We have not carried out experiments of longer duration to test for further methylenation of the cis-10 and cis-11 isomers.

In order to confirm the specificity obtained with the cis-7-16:1 isomer alone, cells were grown overnight on an equimolar mixture of cis-7-16:1 and cis-9-16:1. Gas–liquid chromatography on an open capillary column gave the result illustrated in Figure 3. Two hexadecenoic acid isomers were obtained from the cellular lipids, one with the expected retention of cis-7-16:1 and the other with the expected retention of cis-9-16:1. On the other hand there was only one 17:cyc fatty acid and that had the retention time of *cis*-9,10-methylenehexadecanoate.

Positional Distribution of the Hexadecenoic Acid Isomers on Phosphatidylethanolamine of E. coli. We sought to determine if the specificity of cyclopropane fatty acid formation could be the result of the placement of the hexadecenoic acids on the phospholipids. Phosphatidylethanolamine, the major phospholipid of *E. coli* (Kaneshiro and Marr, 1962), was isolated by thin-layer chromatography and digested with snake venom phospholipase, which is specific for the fatty acid linked to carbon-2 of the *sn*-glycerol 3-phosphate backbone. The ratios of cis-7, cis-9, and cis-10-16:1 on carbon-2 *vs.* carbon-1 are given in Table IV. The three isomers were predominantly located on carbon-2 of phosphatidylethanolamine. The ratio of cis-11-16:1 was also determined and found to be 4.8, but because of the presence of small amounts of 17:cyc-9,10 the exact amounts of 17:cyc-11,12 on carbons-1 and -2 could not be determined without capillary gas–liquid chromatography, which was not used for the measurement of the fatty acids obtained after phospholipase digestion.

Conversion of Octadecenoic Acid Isomers to Cyclopropane Acids. E. coli K1060 was grown on three 18:1 isomers. As has been shown by Silbert *et al.* (1968) with another *E. coli* unsaturated fatty acid auxotroph, both cis-11-18:1 and cis-9-18:1 provided essentially equal growth stimulation. They found that growth with cis-6-18:1 was somewhat delayed and less extensive than with the other two isomers. In our hands with *E. coli* K1060 all three gave similar growth rates, and the final yield was one-third less with cis-6-18:1. The doubling times were from 3.6 to 4.0 hr at 30°. The conversion of the three 18:1 isomers to C_{19}-cyclopropane in overnight cultures is given in Table V. It can be seen that the conversion of cis-6-18:1 to 19:cyc was very low compared to the conversion seen with cis-9-18:1 and cis-11-18:1.

Discussion

The results presented here demonstrate a strong specificity of cyclopropane synthetase *in vivo* for 16:1 fatty acids with

TABLE IV: Positional Distribution of Fed Fatty Acids in Phosphatidylethanolamine.

	C-2[a] / C-1
Cis-7-16:1	8.1
Cis-9-16:1	11.6, 9.0[b]
Cis-10-16:1	7.4

[a] The ratio of the 16:1 isomer plus the cyclopropane acid derived from it, if any, on C-2 of phosphatidylethanolamine divided by the 16:1 + 17:cyc on C-1 of phosphatidylethanolamine. The amount of each fatty acid as its weight percent of the total fatty acids on C-1 or C-2 was the number used in making this calculation. C-1 fatty acids are defined as the fatty acids in lysophosphatidylethanolamine, and C-2 fatty acids are defined as the free fatty acids, after treatment with snake venom phospholipase. [b] See footnote *c* of Table II.

TABLE V: Conversion of Octadecenoic Acid Isomers to Cyclopropane Fatty Acids.

Isomer Fed to Cells	Cellular Fatty Acids 18:1 (Wt % of Total Fatty Acids)	19:cyc (Wt % of Total Fatty Acids)	[19:cyc/(18:1 + 19:cyc)] × 100 (%)
Cis-6-18:1	34.1	0.53	1.5
Cis-9-18:1	28.5	11.3	28.4
Cis-11-18:1	43.8	19.3	30.6

the double bond at least nine carbons from the carboxyl terminus. Neither cis-6 nor cis-7-16:1 was detectably methylenated. Cis-8-16:1 was not examined. Moving toward the methyl terminus, extensive methylenation of cis-10-16:1, but considerably less conversion of cis-11-16:1 was found in overnight cultures (Table II). With exogenous 18:1 isomers, both cis-9-18:1 and cis-11-18:1 were extensively converted to the corresponding cyclopropane fatty acids, as was shown previously by Silbert *et al.* (1968). There was very little conversion of cis-6-18:1 to the corresponding cyclopropane fatty acid.

The results with cis-7-16:1, cis-9-16:1, cis-9-18:1, and cis-11-18:1 parallel previous findings with the natural pairs of isomers found in *Clostridium butyricum* (Goldfine and Panos, 1971). In that organism, 60% conversion of cis-9-16:1, but only 2% conversion of cis-7-16:1 to the corresponding cyclopropane acids was seen in an overnight culture. Of the 18:1 isomers there was 45% conversion of cis-9-18:1 and 79% conversion of cis-11-18:1 to the corresponding cyclopropane acids. These results suggested that the enzyme favors the ω-7 fatty acids. In *E. coli* however, there appears to be little specificity for cis-11-18:1 (ω-7) in comparison to cis-9-18:1 (ω-9). We have not, however, fed the two isomers to *E. coli* K1060 together as was done with cis-7 and cis-9-16:1.

Information on the mechanism of action of cyclopropane synthetase comes mainly from the work of Law and his coworkers who showed that the enzyme from *Clostridium butyricum*, which was partially purified from the soluble fraction, transfers a methyl group from *S*-adenosylmethionine to an unsaturated fatty acid ester linked in any of a number of phospholipids (Zalkin *et al.*, 1963; Chung and Law, 1964; Thomas and Law, 1966). The fatty acid could be linked to either carbon-1 or carbon-2 of *sn*-glycerol 3-phosphorylethanolamine, but carbon-1-linked unsaturated fatty acids appeared to be favored for *in vitro* conversion to cyclopropane fatty acids by the enzyme from *C. butyricum*. It is evident, however, that carbon-2-linked palmitoleic acid can be almost fully converted to the corresponding cyclopropane fatty acid in overnight cultures of *E. coli* (Tables II and IV). Indeed *E. coli* appears to favor carbon-2-linked unsaturated fatty acids. In the experiments summarized in Table IV, a greater proportion of the cis-9-16:1 or cis-10-16:1 on carbon-2 of phosphatidylethanolamine was converted to a cyclopropane acid than that on carbon-1 (data not shown). It should be noted that unsaturated fatty acids tend to be located predominantly on carbon-2 of the phospholipids of *E. coli*, but they are predominantly located on carbon-1 of the diacyl phosphatides in *C. butyricum* (Hildebrand and Law, 1964).

In an investigation of diene formation from monounsaturated fatty acids in *Chlorella vulgaris* and in *Ricinus communis* seeds, and of the formation of hydroxymonounsaturated fatty acids in *R. communis* seeds, a strong specificity for either the cis-9 or the ω-9 monounsaturated fatty acids was observed (Howling *et al.*, 1972). These results were interpreted as suggesting the presence of two desaturases, one of which recognizes the double bond at carbon-9 with respect to the carboxyl end and another which recognizes the double bond at carbon-9 from the methyl terminus. A similar pair of hydroxylases was postulated. Although these reactions are formally different from cyclopropane fatty acid formation, a comparison of the specificities for the position of the double bond in the monounsaturated substrate is of interest. The fatty acids capable of being converted to cyclopropane acids had double bonds at three positions relative to the carboxyl group: cis-9 (16:1 and 18:1), cis-10-16:1, and cis-11 (16:1 and 18:1). Four positions relative to the methyl terminus could be utilized: ω-5 (cis-11-16:1), ω-6 (cis-10-16:1), ω-7 (cis-9-16:1 and cis-11-18:1), and ω-9 (cis-9-18:1). However, another ω-9 acid (cis-7-16:1) was not methylenated. These data can be interpreted as indicating either a series of cyclopropane synthetases of extremely narrow specificities, or, more likely, one or two synthetases with somewhat broader specificities with respect to chain length and the position of the double bond.

In *E. coli*, unsaturated fatty acids are found in both the cytoplasmic membrane and another membrane, which is outside of the rigid peptidoglycan layer (White *et al.*, 1972). It is clear that both the inner and outer membrane unsaturated fatty acids can be converted to cyclopropane acids in overnight cultures, since 93% of cis-9-16:1 was converted in one experiment (Table II). If cyclopropane synthetase of *E. coli* is an enzyme of the cytoplasm as reported by Cronan (1968), there has to be extensive movement of lipids from outer to inner membrane and back for conversion to be as extensive as that observed. In logarithmic cultures of *E. coli* there is a slightly higher proportion of cyclopropane fatty acids in the cytoplasmic membrane than in the outer membrane; however, this is paralleled by a higher proportion of unsaturated fatty acids in the cytoplasmic membranes (Koplow and Goldfine, 1974).

Recent experiments by Cox *et al.* (1973) have demonstrated the presence of cyclopropane synthetase in isolated *E. coli* ML308-225 membrane vesicles, which are substantially free of soluble proteins. It is not clear however, if outer membrane proteins are present in these vesicles, but they are substantially free of lipopolysaccharides when prepared from strain ML308-225 (Kaback, 1972). The question of the exact site of cyclopropane fatty acid formation and possible translocation between membranes is still open.

Our finding of growth-supporting monoenoic acids which are extensively incorporated into phospholipids, but are not converted to cyclopropane fatty acids in *E. coli*, makes possible experiments designed to elucidate the unique roles of unsaturated and cyclopropane fatty acids in bacteria.

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Substrate Enantiomers. Modifiers of Carboxypeptidase A Activity†

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ABSTRACT: The dual specificity of carboxypeptidase A toward ester and peptide substrates was studied by employing substrate enantiomers. The present investigation demonstrates that BzGly-D-OPhe inhibits the hydrolysis of BzGly-L-OPhe. Moreover, dependent on the particular substrate employed, this, and other substrate enantiomers, can activate, inhibit, or leave unaltered the rate of carboxypeptidase-catalyzed hydrolysis. The modes of inhibition have been characterized kinetically, and changes in the circular dichroic spectrum of carboxypeptidase, when labeled with a conformational probe, imply alterations in protein structure consequent to enantiomer binding. The results confirm previous postulates that the catalytic mechanisms of dipeptide and ester hydrolysis by carboxypeptidase include multiple productive and nonproductive enzyme–substrate complexes.

Differences between ester and peptide hydrolysis have long been observed for both native and various modified carboxypeptidases[1] (Vallee *et al.*, 1970). Several years ago a dual-site model, whose basic tenet was multiple nonidentical but overlapping binding sites for esters and peptides, was proposed to account for a number of the observed kinetic phenomena (Vallee *et al.*, 1968). The model suggested that carboxypeptidase could recognize differences between ester and peptide linkages. While this functional group discrimination had been observed only with substrates, it seemed likely that it might also occur with substrate enantiomers. Hence, BzGly-D-OPhe and BzGly-D-Phe, the optical isomers of the most commonly employed substrate pair, were synthesized and their association with carboxypeptidase was examined.

Materials and Methods

Substrates. The synthesis of BzGlyGly-L-Phe has been described (Auld and Vallee, 1970) and that of BzGlyGly-L-OLeu will be described elsewhere (B. Holmquist and D. S. Auld, in preparation). BzGly-L-Phe, from Yeda Chemical Co., and BzGly-L-OPhe, obtained from Fox Chemical Co. and recrystallized from dry acetone, mp 74–75°, were suitable for use.

BzGly-D-OPhe was synthesized from D-Phe according to the method of McClure (1966) and yielded white crystals which were recrystallized from dry acetone and dried *in vacuo* over

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[1] Abbreviations used are: Bz, benzoyl; OPhe, phenyllactate; OLeu, β-isopropyllactate; carboxypeptidase refers to carboxypeptidase A throughout.