THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 240, No. 1, January 1965
Printed in U.S.A.

The Stereospecific Conversion of Stearic Acid to Oleic Acid*

GEORGE J. SCHROEPFER, JR.,† AND KONRAD BLOCH

From the James Bryant Conant Laboratory, Department of Chemistry, Harvard University, Cambridge 38, Massachusetts

(Received for publication, July 13, 1964)

In several enzyme systems that catalyze the desaturation of stearic acid, oleic acid (*cis*-Δ^9-octadecenoic acid) is the sole product and the reaction is therefore characterized by positional and geometrical stereospecificity. We now have found that the desaturating system of *Corynebacterium diphtheriae* has the further property of selectively removing 1 particular hydrogen atom from each pair of hydrogens at carbon atoms 9 and 10 of stearic acid. The enzyme can thus distinguish between the 2 hydrogen atoms attached to a carbon atom of a polymethylene chain. The present report is the first on the stereochemistry of the biological introduction of an isolated double bond into an acyclic compound, and represents a notable example of the stereospecificity of enzymatic reactions at meso carbon atoms (1). Part of this work has been published in preliminary form (2, 3).

EXPERIMENTAL PROCEDURE AND RESULTS

Experimental Approach

In order to investigate the stereospecificity of the enzymatic conversion of stearic acid to oleic acid, we needed stearic acid samples labeled singly and stereospecifically with isotopic hydrogen. The approach which seemed to us most promising was to prepare optically active 9- and 10-hydroxystearic acids and their enantiomorphs and to replace the hydroxyl function of all four acids with tritium by stereospecific chemical reactions. These transformations can be effected by treating the tosylates of the hydroxystearates with isotopically labeled lithium aluminum hydride as shown in Fig. 1. This type of reaction has been shown to proceed with an inversion of configuration (4–6). Thus, treatment of the tosylate of 9-D-hydroxystearate with tritium-labeled lithium aluminum hydride was expected to yield 9-L-tritiostearic acid, and 9-D-tritiostearic was expected from the hydrogenolysis of the tosylate of the 9-L-hydroxystearate (after oxidation of the resulting octadecanols). Analogous reactions should afford two stearic acids labeled stereospecifically at carbon atom 10. Since some racemization is to be expected in the hydrogenolysis of tosylates (5), the resultant tritium-labeled stearic acids will be partially racemized with respect to tritium, and the enzymatic conversion to oleic acid, if stereospecific, will not proceed with complete removal or retention of labeled hydrogen from a given position.

The desired 9- and 10-hydroxystearic acids, derived from natural sources, have been described by others (7, 8). Moreover, Baker and Gunstone (9) have recently reported the chemical synthesis of the D isomer of 9-hydroxystearic acid. None of these hydroxystearic acids showed measurable optical activity. While the chemical synthesis of the stereoisomers of 9- and 10-hydroxystearic acids is feasible, it was considered less laborious to prepare the readily available hydroxy acids derived from natural sources and to demonstrate unequivocally that they are optically active. Chemical inversion of the hydroxyl functions would yield the corresponding enantiomorphs (Fig. 2). These pairs of 9- and 10-hydroxystearic acids could then be used to prepare the four mono-^{3}H-stearic acids, each labeled stereospecifically at a single position.

As a source of the enzyme for the conversion of stearic acid to oleic acid, growing cultures of a strain of *C. diphtheriae* (10) offer several advantages over other available systems. The uptake and desaturation of added stearic acid by this organism are very efficient. Preliminary preparation of the coenzyme A derivative is not necessary. Little or no degradation of the stearic acid by the organism occurs under the conditions employed (10). Moreover, the low degree of dilution of added labeled stearic acid and of the oleic acid formed from it by their endogenous counterparts facilitates the isolation of sufficient amounts of the two compounds for accurate radioactivity measurements and, in studies employing deuterium-labeled substrates, makes feasible direct deuterium analysis of the fatty acids by mass spectrometry.

General Procedures

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Optical rotations were measured in a Rudolph spectropolarimeter[1] at 23 ± 1°. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. Stearic acid-1-^{14}C, purified by mercuric acetate treatment and silicic acid column chromatography, was a gift from Dr. A. J. Fulco. The radiopurity of this material was in excess of 97% as judged by silicic acid column chromatography and vapor phase chromatography. Infrared spectra were recorded on a Beckman IR-5A spectrometer with KBr micropellets.

Preparation of Hydroxystearic Acids

(−)-*Methyl 9-D-Hydroxyoctadecanoate*—9-Hydroxyoctadecanoic acid, prepared by catalytic reduction of 9-hydroxy-Δ^{12}-octade-

* Supported by grants-in-aid from the National Science Foundation, the United States Public Health Service, and the Eugene P. Higgins Trust Fund of Harvard University.

† Recipient of a Research Career Development Award from the National Heart Institute, United States Public Health Service. Most of this work was carried out while on leave of absence from the Department of Biochemistry, University of Minnesota Medical School. Present address, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois.

[1] We are indebted to Professor P. Doty for the use of this instrument, and to Dr. P. Urnes for instruction in its operation.

cenoic acid (7) from *Strophanthus* oil, was a generous gift from Dr. A. J. Fulco. The methyl ester, prepared by treatment of the acid with diazomethane (11), was purified by silicic acid column chromatography and recrystallized from methanol. The melting points of the free acid and of the methyl ester are listed in Table I. The methyl ester was found to be levorotatory (Table II). On recrystallization of the ester from methanol, the rotational values remained essentially constant.

Baker and Gunstone were unable to measure the optical activity of their synthetic 9-D-hydroxyoctadecanoic acid or of a sample of the acid prepared from the naturally occurring Δ^{12}-9-hydroxyoctadecenoic acid. However, they assigned the D configuration to the acid derived from the olefinic hydroxy acid on the basis of mixture melting point data (9). Through a generous gift of synthetic 9-D-hydroxyoctadecanoic acid from Professor Gunstone, we have been able to show that the methyl ester (prepared by treatment with diazomethane and purified by silicic acid column chromatography and recrystallization from methanol) of his synthetic acid is levorotatory (Table II), thus confirming the assignment of the D configuration to the "natural" 9-hydroxyoctadecanoic acid.

(+)-*Methyl 9-L-Hydroxyoctadecanoate*—To (−)-methyl 9-D-hydroxyoctadecanoate (0.584 g) in pyridine (35 ml, dried over barium oxide) cooled to −10° was added *p*-toluenesulfonyl chloride (3.34 g, recrystallized from ether). The reaction mixture was kept at −10° for 1 week and for another week at 4°. After the slow addition of 70 ml of cold water, the solution was extracted three times with ether. The ether solution was washed three times with water and dried over anhydrous magnesium sulfate. Evaporation of the solvent under nitrogen yielded 0.66 g of a yellowish oil. The tosylate was purified by column chromatography on activated silicic acid (50 g, Clarkson Chemical Company). Elution with 5% ether in pentane (200 ml) yielded 21 mg of an oily material which partially solidified on standing at room temperature. This material was not studied further. Further elution with 5% ether in pentane (200 ml) yielded no detectable material on evaporation of the solvent. The tosylate (0.614 g) was isolated as a colorless, viscous oil by elution with 15% ether in pentane. The tosylate (0.497 g) was heated under reflux with stirring for 10 hours with a mixture of 4 N NaOH (50 ml) and dioxane (10 ml). Water and ether were added, and the ether layer was discarded. The aqueous phase was acidified to pH 1 with cold 6 N sulfuric acid and extracted three times with ether. The ethereal solution was dried over anhydrous magnesium sulfate and filtered, and the ether was evaporated under nitrogen. The acids were separated into nonpolar and polar fractions on a column of activated silicic acid. Elution with 10% ether in pentane yielded 0.115 g of an oil which solidified on standing at room temperature. This material was not studied further, nor was the fraction eluted with 30% ether in pentane (0.014 g). Elution with 50% ether in pentane yielded a white solid (0.175 g) which was methylated by treatment with diazomethane and rechromatographed on an activated silicic acid column. Elution with 10% ether in pentane and with 15% ether in pentane yielded 0.02 g of oily material. The 9-hydroxy fatty acid methyl ester (0.119 g) was eluted with 30% ether in pentane. The melting points of the methyl ester (recrystallized from methanol) and of the free acid (after saponification of the methyl ester and recrystallization from Skellysolve B and acetone-water) are listed in Table I. The methyl ester was dextrorotatory (Table II). The optical purity of the ester

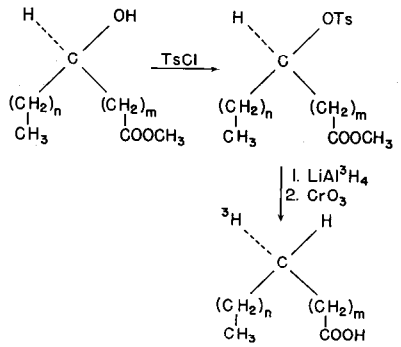

FIG. 1. Stereospecific syntheses of tritium-labeled stearic acids from optically active methyl 9-hydroxyoctadecanoate (n = 8, m = 7) or 10-hydroxyoctadecanoate (n = 7, m = 8). Applied to the two enantiomorphic pairs of hydroxy acids, this procedure yields four stereospecifically labeled monotritiostearic acids. *TsCl*, *p*-toluenesulfonyl chloride.

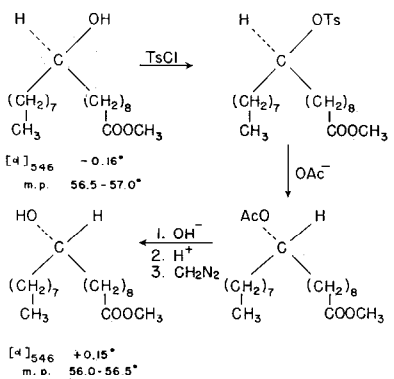

FIG. 2. Preparation of dextrorotatory methyl 10-hydroxyoctadecanoate from levorotatory methyl 10-hydroxyoctadecanoate. Analogous inversion reactions afford (+)-methyl 9-L-hydroxyoctadecanoate from (−)-methyl 9-D-hydroxyoctadecanoate. *TsCl*, *p*-toluenesulfonyl chloride.

TABLE I

Melting points of hydroxy fatty acids

Compound	Observed	Recorded	Reference
(−)-Methyl 9-D-hydroxyoctadecanoate ("natural")	53.0–53.5°		
Free acid	82.0–83.5	81–82°	9
(−)-Methyl 9-D-hydroxyoctadecanoate (synthetic)			
Free acid		83.5–84.5	9
(±)-Methyl 9-DL-hydroxyoctadecanoate		50.0–51.5	12
Free acid		76–77	12
(+)-Methyl 9-L-hydroxyoctadecanoate	51.0–52.5		
Free acid	82.0–84.0		
(−)-Methyl 10-hydroxyoctadecanoate	56.5–57.0	53–54	8
Free acid	86.5–87.5	86.0–86.5	8
(+)-Methyl 10-hydroxyoctadecanoate	56.0–56.5		
Free acid	85.0–86.0		
(±)-Methyl 10-DL-hydroxyoctadecanoate		54–55	12
Free acid		79–80	12

TABLE II

Optical activity of 9-hydroxy fatty acid methyl esters

All rotations were measured in methanol. Path length was 2 dm. Sample I, (−)-methyl 9-D-hydroxyoctadecanoate ("natural"), c = 5.61; Sample II, I, recrystallized from methanol, c = 2.60; Sample III, (−)-methyl 9-D-hydroxyoctadecanoate (synthetic (9)), c = 3.62; Sample IV, (+)-methyl 9-L-hydroxyoctadecanoate, prepared from tosylate of II, c = 3.27.

Wave length	Specific rotation ([α] ± s.e. (s.d.))			
	Sample I	Sample II	Sample III	Sample IV
mμ				
546	−0.18 ± 0.01° (0.04)	−0.12 ± 0.01° (0.03)	−0.17 ± 0.02° (0.05)	+0.17 ± 0.01° (0.04)
436	−0.29 ± 0.01 (0.03)	−0.31 ± 0.02 (0.05)	−0.29 ± 0.02 (0.05)	+0.21 ± 0.02 (0.04)
408	−0.37 ± 0.01 (0.04)	−0.37 ± 0.02 (0.05)		
365	−0.53 ± 0.01 (0.06)	−0.41 ± 0.02 (0.05)	−0.60 ± 0.02 (0.05)	+0.37 ± 0.02 (0.04)
313		−0.71 ± 0.02 (0.04)		

TABLE III

Optical activity of 10-hydroxy fatty acid methyl esters

For experimental conditions, see Table II.

Wave length	Specific rotation ([α] ± s.e. (s.d.))	
	(−)-Methyl 10-hydroxyoctadecanoate (c = 2.16)	(+)-Methyl 10-hydroxyoctadecanoate (c = 3.92)
mμ		
546	−0.16 ± 0.03° (0.07)	+0.15 ± 0.02° (0.05)
436	−0.32 ± 0.02 (0.05)	+0.24 ± 0.01 (0.04)
408	−0.39 ± 0.02 (0.08)	
365	−0.51 ± 0.02 (0.08)	+0.40 ± 0.02 (0.05)
313	−0.83 ± 0.02 (0.08)	

was estimated to be approximately 89%, based on comparison of the total of the rotation values of the dextrorotatory methyl ester at 546, 436, and 365 mμ with the total of the averages of the rotation values obtained in the three measurements on the levorotatory methyl ester samples at the same wave lengths. The tosylate of the (+)-methyl 9-L-hydroxyoctadecanoate was prepared and purified as described for the 9-D-hydroxy compound.

(−)-*Methyl 10-Hydroxyoctadecanoate*—10-Hydroxyoctadecanoic acid was prepared by incubating oleic acid with a fermenting culture of a *Pseudomonas* species (NRRL-B-2994)[2] under the conditions described by Wallen, Benedict, and Jackson (8). The melting points of the acid and of the methyl ester are listed in Table I. Wallen *et al.* suspected that the hydroxy acid was optically active, but they were unable to measure the rotation. The methyl ester of the 10-hydroxyoctadecanoic acid produced from oleic acid by the microorganism is levorotatory (Table III).

(+)-*Methyl 10-Hydroxyoctadecanoate*—The tosylate of (−)-methyl 10-hydroxyoctadecanoate was prepared and purified essentially as outlined for the 9-D-hydroxy compound (90% yield). In an attempt to reduce the racemization encountered in the inversion of the 9-hydroxy fatty acid ester, the following procedure was used. The tosylate (0.80 g) was treated with 0.287 g of anhydrous sodium acetate in 40 ml of acetic acid for 24 hours at 60°. Water was added, and the resulting solution was extracted three times with ether. The combined ethereal solutions were washed four times with water and dried over anhydrous magnesium sulfate. After evaporation of the solvent the resulting yellowish oil was heated for 3 hours with a solution of 15 g of KOH in 60 ml of 25% methanol. After acidification to pH 1 with dilute HCl, the solution was extracted three times with ether, and the combined ether solutions were washed three times with water and dried over anhydrous magnesium sulfate. The fatty acids were then methylated with diazomethane and chromatographed on an activated silicic acid column (50 g) as described for the 9-hydroxy compound. The yield was 0.222 g of the 10-hydroxy fatty acid methyl ester. The melting points of the methyl ester (recrystallized from methanol) and of the free acid (prepared from the methyl ester and recrystallized from acetone) are recorded in Table I. The methyl ester was dextrorotatory (Table III). The optical purity was estimated to be about 89%, based on comparison of the total of the rotations of the dextrorotatory methyl ester at 546, 436, and 365 mμ with the total of the rotations of the levorotatory methyl ester at the same wave lengths. The tosylate of the dextrorotatory ester was prepared and purified as described for 9-D-hydroxyoctadecanoate.

The chemical identity of each of the hydroxystearates is based on (*a*) melting points of the methyl ester and of the free acid (Table I), (*b*) chromatographic properties on silicic acid columns, (*c*) behavior upon vapor phase chromatographic analysis on a variety of columns including diethylene glycol succinate polyester and SE-30 silicone columns, (*d*) infrared spectra of the methyl esters and of the free acids (Fig. 3), and (*e*) the source and mode of preparation of these compounds. The chemical purity of these hydroxystearates was greater than 97% as judged by the above techniques.

The absolute values of the optical rotations of the hydroxystearates are extremely small. The possibility that the observed rotations were due to impurities can be ruled out by the following observations. (*a*) The magnitude of the optical rotations did not change significantly on recrystallization. (*b*) The rotations of both the 9- and 10-hydroxystearates were comparable in magnitude, and they were also comparable in magnitude to the rotation observed with a sample of chemically synthesized 9-D-hydroxystearate. (*c*) The "natural" 9- and 10-hydroxystearates were converted to their enantiomorphs by standard chemical reactions, and the magnitudes of the rotations of the inversion products were comparable (with allowance for some expected racemization during their formation by hydrolysis or acetolysis of the tosylates) to those observed in the isomers from which they had been prepared.

Stearic Acids Labeled Stereospecifically with Tritium—The tosylates of the hydroxy fatty acid methyl esters (7 to 8 μmoles)

[2] We wish to thank Dr. L. L. Wallen for a gift of a culture of this organism.

were heated under reflux for 20 to 26 hours with tritium-labeled lithium aluminum hydride (10 mc, 95 μmoles, New England Nuclear Corporation) in tetrahydrofuran (8 ml, freshly distilled from lithium aluminum hydride). Excess hydride was decomposed with moist tetrahydrofuran. Dilute HCl, water, and ether were successively added. The ether layer was separated, and the aqueous phase was extracted twice more with ether. The combined ether solutions were washed five times with water and dried over anhydrous magnesium sulfate. The tritium-labeled stearyl alcohol was mixed with carrier (10 to 11 mg, Mann Research Laboratories) and purified by chromatography on a silicic acid (Mallinckrodt Chemical Works)-Super-Cel (Johns Manville) (2:1) column with benzene as the eluting solvent. The

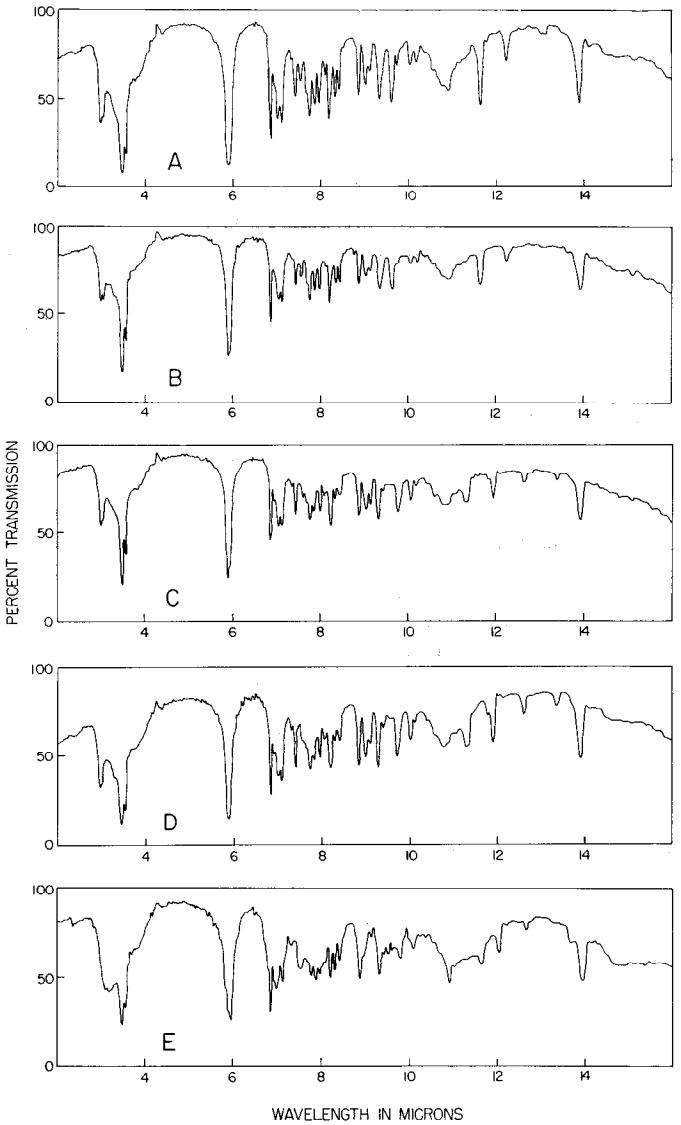

FIG. 3. Infrared spectra of 9-, 10-, and 12-hydroxyoctadecanoic acids. *A*, 9-D-hydroxyoctadecanoic acid; *B*, 9-L-hydroxyoctadecanoic acid; *C*, 10-D-hydroxyoctadecanoic acid; *D*, 10-L-hydroxyoctadecanoic acid; *E*, 12-D-hydroxyoctadecanoic acid. (See the text regarding assignments of configuration to these compounds.) While the infrared spectra of the 9-D- and 10-D-hydroxyoctadecanoic acids are essentially identical with those of their enantiomorphs, there are significant differences in the spectra of the 9-, 10-, and 12-hydroxyoctadecanoic acids in the 7 to 10 μ region.

TABLE IV

^3H-Stearic acids

Compound	Melting point	Specific activity
		μc/mg
9-D-^{3}H-Stearic acid	69.0–70.0°	0.21
9-L-^{3}H-Stearic acid	69.5–70.5	0.28
10-^{3}H-Stearic acid (from tosylate of (−)-10-hydroxyoctadecanoate)	69.5–70.5	0.15
10-^{3}H-Stearic acid (from tosylate of (+)-10-hydroxyoctadecanoate)	70.2–71.2	0.08

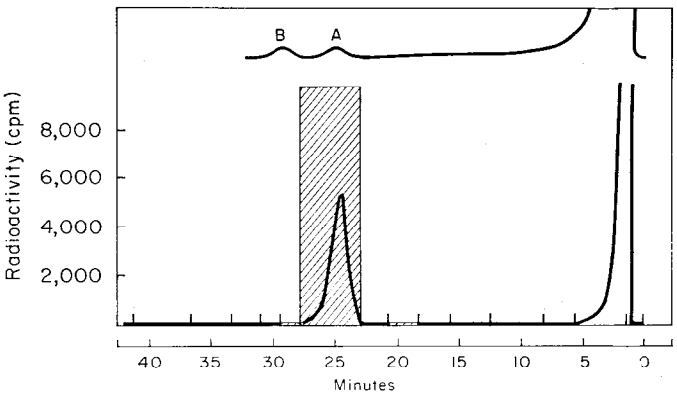

FIG. 4. Vapor phase chromatographic analysis of purity of methyl 9-L-^{3}H-stearate. The *lower tracing* indicates that most of the radioactivity (*hatched bars*) collected at the exit of the column was associated chromatographically with methyl stearate. The *upper tracing* illustrates the separation of methyl stearate (*A*) and methyl oleate (*B*) under the same experimental conditions.

stearyl alcohol was further diluted with 36 to 38 mg of carrier and oxidized by chromic acid under the conditions described by Pattison, Stothers, and Woolford (13). After 22 to 24 hours the reaction mixture was diluted with water, and the crude stearic acid was collected on a filter and washed several times with water. To remove any residual stearyl alcohol and readily exchangeable tritium, the acid was heated in aqueous methanolic KOH; the alkaline solution was washed with pentane, acidified, and extracted with ether; and the ether solution was washed several times with water. The stearic acids were recrystallized from Skellysolve B and acetone-water. The melting points and specific activities of the four tritium-labeled stearic acids are listed in Table IV.

The purity of each of the stearic acids was judged to be in excess of 97% on the basis of vapor phase chromatographic analysis of the methyl esters. The radiopurity of the tritium-labeled acids was greater than 90% in all cases. This statement is based upon two types of tests. First, the methyl ester of each of the stearic acids was subjected to vapor phase chromatography. Effluent from the column was collected and assayed for radioactivity (14). Fig. 4 shows the results for 9-L-^{3}H-stearic acid. Virtually all of the radioactivity collected at the exit of the column was associated chromatographically with methyl stearate. Since the collection of radioactivity was not quantitative, the presence of significant amounts of more polar radioactive impurities cannot be excluded by this type of analysis. In order to rule out this possibility, a small amount of stearic acid-1-^{14}C of high radiopurity was added to each of the four tritium-labeled

TABLE V

Radiochemical purity of tritium-labeled stearic acids

Each of the tritium-labeled acids was mixed with a small amount of stearic acid-1-^{14}C of high radiochemical purity (greater than 97%). One or more aliquots were taken for assay of radioactivity. The remainder of the material was methylated by treatment with diazomethane and subjected to vapor phase chromatography on 10% diethylene glycol succinate polyester columns. The methyl stearate was collected at the exit of the column and assayed for radioactivity. The unchanged ^{3}H:^{14}C ratio is taken as indicative of a high state of purity of the tritium-labeled acid.

Compound	^{3}H	^{14}C	^{3}H:^{14}C	^{3}H:14C*
	c.p.m.	*c.p.m.*		
9-L-^{3}H-Stearic acid-1-^{14}C	12,750	3,340	3.82	1.00
Methyl 9-L-^{3}H-stearate-1-^{14}C	7,610	2,110	3.60	0.94
9-D-^{3}H-Stearic acid-1-^{14}C	12,560	3,270	3.84	1.00
Methyl 9-D-^{3}H-stearate-1-^{14}C	7,980	2,100	3.80	0.99
10-^{3}H-Stearic acid-1-^{14}C (from tosylate of (−)-methyl 10-hydroxyoctadecanoate)	1,210	880	1.37	1.00
	1,200	920	1.30	
Methyl 10-^{3}H-stearate-1-^{14}C	1,010	823	1.23	0.92
	250	206	1.21	0.90
10-^{3}H-Stearic acid-1-^{14}C (from tosylate of (+)-methyl 10-hydroxyoctadecanoate)	825	581	1.42	1.00
	805	545	1.48	
Methyl 10-^{3}H-stearate-1-^{14}C	248	175	1.42	0.98
	365	247	1.48	1.02
	718	502	1.43	0.99
10-^{3}H-Stearic acid-1-^{14}C (from tosylate of (+)-methyl 10-hydroxyoctadecanoate)	896	967	0.927	
	875	948	0.923	1.00
	866	947	0.914	
	891	962	0.926	
Methyl 10-^{3}H-stearate-1-^{14}C	863	968	0.892	0.97
	961	1,070	0.900	0.98

* For ease of comparison, the last column lists the ^{3}H:^{14}C ratios relative to an assigned value of unity for the stearic acids before purification.

acids. An aliquot was taken for simultaneous assay of ^{3}H and ^{14}C. Each stearic acid was then methylated and subjected to vapor phase chromatographic analysis. Methyl stearate was collected from the exit of the column and assayed for ^{3}H and ^{14}C. An unchanged ^{3}H:^{14}C ratio is indicative of a high state of purity of the tritium-labeled acid. The results of these experiments are shown in Table V. By these criteria all four of the stearic acids were considered sufficiently pure for the enzymatic studies.

Microbiological Desaturation of Stearic Acids Labeled Stereospecifically with Tritium—After the addition of a small amount of stearic acid-1-^{14}C, each of the tritium-labeled acids was dissolved in absolute ethanol. An aliquot was taken for subsequent assay of radioactivity. The ethanolic solutions (0.6 to 2.0 ml) were added to 500 or 1000 ml of the culture medium of Mueller and Miller (15), supplemented with L-tryptophan (0.1 g per liter), L-glutamic acid (0.5 g per liter), and maltose (20 g per liter). The media were inoculated with suspensions of *C. diphtheriae* strain C_7 s(−) tox(−) (16), kindly provided by Professor A. M. Pappenheimer. The bacteria were allowed to grow in the presence of the labeled acids for 22 to 26 hours at 30° on a rotating shaker. The bacteria were collected by centrifugation, washed with water, and weighed. After digestion of the bacteria for 3 hours in a refluxing solution of 20% KOH in 50% methanol and subsequent acidification of the digest to pH 1, the fatty acids were extracted four times with ether. The combined ether solutions were washed with water and dried over anhydrous magnesium sulfate. The methyl esters were prepared by treatment of the acids with diazomethane and subjected to chromatography on an activated silicic acid column to which a small amount of activated charcoal was added to remove a yellow pigment. The methyl esters of the fatty acids were eluted from the column with 5% ether in pentane. After evaporation of the solvent, the esters were separated into saturated and unsaturated fatty acid ester fractions by treatment with 2.0 g of mercuric acetate in 50 ml of refluxing methanol for 2 hours. After complete evaporation of the methanol, the residual material was applied to a silicic acid column (30 g). Saturated esters were eluted with 200 ml of 5% ether in pentane. Further elution with 100 ml of the same solvent mixture yielded 1 to 2% of the ^{14}C activity eluted in the previous 200-ml fraction, indicating that elution of the

TABLE VI

Microbiological desaturation of tritium-labeled stearic acids

Substrate	Experiment	Substrate	Volume of culture	Time of incubation	Wet weight of bacteria	Total fatty acid methyl esters	Unsaturated esters*	Uptake of added substrate	Extent of desaturation†
		mg	*ml*	*hrs*	*g*	*mg*	%	%	%
9-D-^{3}H-Stearic acid-1-^{14}C	1	2.08	1000	22	9.3	130	28	74	56
	2	1.25	500	23	10.6	114	29	54	48
9-L-^{3}H-Stearic acid-1-^{14}C	1	2.12	1000	22	9.7	132	33	74	57
	2	1.15	500	23	10.0	126	28	60	41
10-^{3}H-Stearic acid-1-^{14}C (prepared from tosylate of (−)-10-hydroxyoctadecanoate)	1	0.85	1000	26	9.7	120	30	72	35
10-^{3}H-Stearic acid-1-^{14}C (prepared from tosylate of (+)-10-hydroxyoctadecanoate)	1	1.21	1000	26	9.6	115	35	69	32
	2	1.96	500	24	8.7	122	39	72	58
	3	1.96	500	24	6.6	78	33	65	55

* Percentage of unsaturated esters $= \frac{\text{mg unsaturated esters}}{\text{mg total cellular fatty acid methyl esters}} \times 100.$

† Percentage of desaturation $= \frac{\text{c.m.p. } ^{14}\text{C in unsaturated fatty acids}}{\text{c.p.m. } ^{14}\text{C in total cellular fatty acids}} \times 100.$

saturated esters was virtually complete. The mercuric acetate adducts of the unsaturated fatty acid methyl esters were eluted with 200 ml of a 1% solution of acetic acid in methanol. After the addition of an equal volume of ether and 5 to 10 ml of water, 13 ml of concentrated HCl were added to decompose the complexes. The mixture was diluted with water and the ether layer was removed. The aqueous phase was extracted once more with ether, and the combined ether solutions were washed four times with water. After drying over anhydrous magnesium sulfate, the unsaturated ester fraction was treated again with diazomethane and purified by silicic acid chromatography. The saturated and unsaturated fatty acid methyl esters were weighed and assayed for radioactivity. Table VI lists the percentage of the fatty acids taken up by the bacteria from the culture medium, the extent of desaturation, and other pertinent information.

The amounts of tritium relative to ^{14}C in the saturated and unsaturated fatty acid methyl esters are listed in Tables VII and VIII along with the amounts of tritium relative to ^{14}C in methyl stearate and methyl oleate which were isolated from these fractions by vapor phase chromatography. The presence of ^{14}C in the fatty acid samples provides an internal standard for determining the extent of tritium removal in the desaturation reaction. The calculations are therefore independent of the degree of dilution of substrate and reaction product by endogenous fatty acid.

The results obtained after incubating the two 9-^{3}H-labeled substrates (Table VII) show that on conversion of oleic acid, tritium in the 9-D-configuration of stearic acid was extensively removed and tritium in the 9-L configuration was retained. Thus, the enzymatic hydrogen abstraction from carbon atom 9 of stearic acid is a stereospecific process.

TABLE VII

Enzymatic desaturation of stearic acids labeled stereospecifically with tritium at carbon atom 9

Compound	^{3}H	^{14}C	^{3}H:^{14}C	^{3}H:^{14}C*
	c.p.m.	*c.p.m.*		
9-D-^{3}H-Stearic acid-1-^{14}C				
Experiment 1				
Substrate	7,300	1,930	3.78	1.00
	7,280	1,930	3.77	
Saturated esters	50,000	8,280	6.04	1.60
Methyl stearate	2,290	382	5.99	1.58
Unsaturated esters	3,090	1,800	1.72	0.46
Methyl oleate	2,380	1,810	1.31	0.35
Experiment 2				
Substrate	12,600	3,270	3.85	1.00
Saturated esters	18,900	3,740	5.05	1.31
Methyl stearate	1,420	303	4.69	1.22
Methyl oleate	3,110	2,580	1.21	0.31
9-L-^{3}H-Stearic acid-1-^{14}C				
Experiment 1				
Substrate	4,280	787	5.44	1.00
	2,690	511	5.26	
Saturated esters	44,100	7,820	5.64	1.05
Methyl stearate	3,800	697	5.45	1.02
Unsaturated esters	8,770	1,810	4.85	0.91
Methyl oleate	4,340	908	4.78	0.89
Experiment 2				
Substrate	12,800	3,340	3.83	1.00
Saturated esters	17,200	4,600	3.74	0.98
Methyl stearate	892	246	3.63	0.95
Methyl oleate	8,080	2,270	3.56	0.93

* For ease of comparison, the last column lists the ^{3}H:^{14}C ratios relative to an assigned value of unity for the substrate.

TABLE VIII

Enzymatic desaturation of stearic acids labeled stereospecifically with tritium at carbon atom 10

	^{3}H	^{14}C	^{3}H:^{14}C	^{3}H:^{14}C*
	c.p.m.	*c.p.m.*		
10-^{3}H-Stearic acid-1-^{14}C (prepared from tosylate of (+)-10-hydroxyoctadecanoate)				
Experiment 1				
Substrate	896	967	0.93	
	875	948	0.92	1.00
	866	947	0.91	
	891	962	0.93	
Saturated esters	2140	2240	0.96	1.04
Methyl stearate	537	588	0.91	0.99
Unsaturated esters	194	1050	0.19	0.21
	70	388	0.18	0.20
Methyl oleate	34	430	0.079	0.09
Experiment 2				
Substrate	825	581	1.42	1.00
	805	545	1.48	
Saturated esters	2090	1450	1.44	0.99
Methyl stearate	509	355	1.43	0.99
Unsaturated esters	602	2090	0.29	0.20
Methyl oleate	134	899	0.15	0.10
Experiment 3				
Substrate	825	581	1.42	1.00
	805	545	1.48	
Saturated esters	2090	1450	1.44	0.99
Methyl stearate	605	417	1.45	1.00
Unsaturated esters	521	1780	0.29	0.20
Methyl oleate	141	868	0.16	0.11
10-^{3}H-Stearic acid-1-^{14}C (prepared from tosylate of (−)-10-hydroxyoctadecanoate)				
Experiment 1				
Substrate	1210	883	1.37	1.00
	1200	922	1.30	
Saturated esters	2550	2000	1.28	0.96
Methyl stearate	284	249	1.14	0.85
Unsaturated esters	999	797	1.25	0.93
Methyl oleate	487	415	1.17	0.87

* Same as in Table VII.

Removal of hydrogen from carbon atom 10 of stearic acid on conversion to oleic acid was also stereospecific. In this case tritium in one configuration was removed while tritium in the opposite configuration was not (Table VIII). Evidence that the hydrogen removed from carbon atom 10 also has the D configuration is presented below.

While complete removal or retention of labeled hydrogen on conversion of the stearates to oleic acid was not observed, the extent of the differences between the results obtained with these substrates and the results which would be expected for complete removal or retention of labeled hydrogen is in accord with the amount of racemization expected in the reactions involved in the preparation of these substrates.

Tritium and ^{14}C assays of the unmetabolized stearic acids remaining at the end of the incubation showed significant enrich-

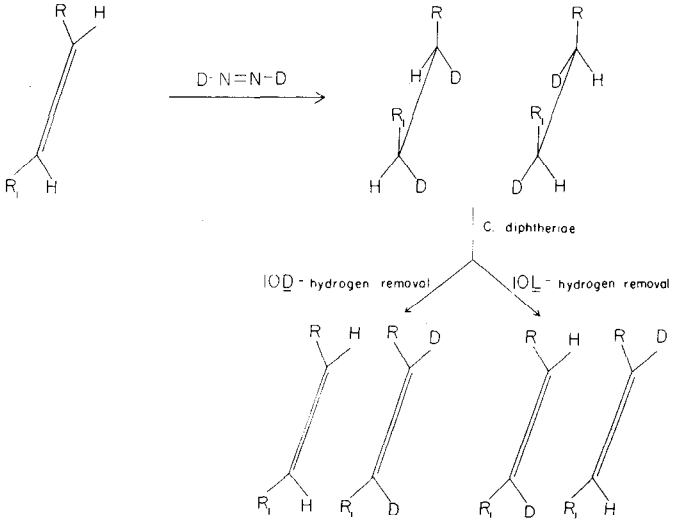

FIG. 5. Stereospecific synthesis of 9,10-dideuteriostearic acid. R = $-(CH_2)_7-COOH$; R_1 = $-(CH_2)_7-CH_3$. Also illustrated are the types of oleic acid molecules expected from enzymatic desaturation involving stereospecific removal of the 9-D hydrogen and of either the 10-D hydrogen atom or the 10-L hydrogen atom.

ment with tritium relative to ^{14}C in the single case of 9-D-^{3}H-stearic acid, a substrate which loses tritium on desaturation (Table VII). The ^3H:^{14}C ratio remained constant in the 10-^{3}H-stearic acid substrate from which tritium was removed upon enzymatic conversion to oleic acid (Table VIII). No enrichment with tritium was found in the two stearate samples which retain tritium on desaturation (Tables VII and VIII). These findings are compatible with the presence of a kinetic isotope effect during removal of the 9-D hydrogen atom. The magnitude of this effect cannot be calculated from the available data. However, the findings suggest that in the desaturation reaction the initial rate-limiting step is the removal of the 9-D hydrogen, and that this is followed by stereospecific removal of 1 of the hydrogen atoms at carbon atom 10.

Identity of Reaction Product as Oleic Acid—According to Fulco, Levy, and Bloch (10), the desaturation product of stearate in the same strain of *C. diphtheriae* is a Δ^9-octadecenoic acid as shown by catalytic reduction of the olefinic acid to stearate and by oxidative degradations to locate the double bond. The predominant unsaturated fatty acid in this bacterium is a Δ^9-hexadecenoic acid which lacks any absorption at 10.3 μ and therefore has the *cis* configuration. That the Δ^9-octadecenoic acid of *C. diphtheriae* also has the *cis* configuration was established in the following way. A culture of the organism was grown in the presence of added stearic acid, and the C_{18} monounsaturated fatty acid was isolated by vapor phase chromatography of the unsaturated fatty acid methyl esters as described above. After a preliminary purification by silicic acid column chromatography, the methyl ester was subjected to thin layer chromatography as described by Morris (17). In this system complete separation of standard samples of methyl oleate and methyl elaidate is achieved. The C_{18} Δ^9-monounsaturated fatty acid methyl ester from *Corynebacterium* yielded a single spot on the chromatogram with the same R_F as that of a standard sample of methyl oleate.

Absolute Configuration of Hydrogen Removed from Carbon Atom 10 upon Enzymatic Conversion of Stearic Acid to Oleic Acid—The 10-^{3}H-stearic acid from which tritium was removed upon enzymatic conversion to oleic acid was prepared from the tosylate of the (+)-methyl 10-hydroxystearate. If the absolute configuration of the 10-hydroxystearate were known, the configuration of the tritium in the stearic acid derived from this hydroxystearate could be specified. From rotational analogies it can be inferred that the levorotatory 10-hydroxystearate produced by the pseudomonad from oleic acid is of the D configuration, since the 9- and 12-hydroxystearates[3] of the D configuration are also levorotatory under the same conditions. By the same token the dextrorotatory inversion product would have the L configuration. Acceptance of this argument would lead to the conclusion that the tritium in the stearic acid formed—with inversion—from the tosylate of the dextrorotatory methyl 10-hydroxystearate is of the D configuration. Since the assignments of configurations on the basis of rotational analogies can be only tentative, a more definitive solution to this problem was sought. Chemical synthesis of 10-D-hydroxystearic acid by the approach introduced by Serck-Hanssen (19, 20) is feasible and would conclusively establish the configurations of the 10-hydroxystearic acids. Equally feasible, more convenient, and, in addition, capable of yielding confirmatory evidence for the stereospecific nature of the desaturation reaction is the following procedure.

Corey, Pasto, and Mock (21) have shown that addition of deuterium to olefins from deuterium-labeled diimide proceeds in a highly stereospecific manner. For example, reduction of fumaric and maleic acids occurs stereospecifically with *cis* addition of deuterium. Reduction of oleic acid, a *cis*-olefin, with deuterium-labeled diimide should yield, by *cis* addition of deuterium, an equal number of two species of molecules, 9-D-$^2H_1$-10-D-$^2H_1$-stearic acid and 9-L-$^2H_1$-10-L-$^2H_1$-stearic acid (Fig. 5). The above studies with the tritium-labeled stearic acids have shown that the 9-D-hydrogen and one, so far unspecified, hydrogen at carbon atom 10 are stereospecifically removed upon enzymatic conversion to oleic acid. The use of stearic acid labeled stereospecifically with deuterium at carbon atoms 9 and 10, as indicated above, can distinguish the two following situations: (*a*) stereospecific removal of the 10-D hydrogen; *i.e.* the hydrogens removed from carbon atoms 9 and 10 have the same absolute configuration; and (*b*) stereospecific removal of the 10-L hydrogen; *i.e.* the hydrogens removed from carbon atoms 9 and 10 have the opposite configurations. Analysis of the oleic acid by mass spectrometry should give the following results for cases *a* and *b*, respectively.

Hydrogen removal	Per cent of oleic acid molecules		
	Dideuterated	Monodeuterated	Undeuterated
(*a*) 10-D	50	0	50
(*b*) 10-L	0	100	0

These predictions are based on the following assumptions. (*a*) The stearic acid substrate is fully dideuterated and the chemical introduction of deuterium is fully stereospecific. (*b*) The added stearic acid or the oleic acid formed from it is not diluted by its endogenous counterpart in the bacterial system. (*c*) Both

[3] 12-D-Hydroxystearic acid (m.p. 79.5–81.0°; infrared spectrum shown in Fig. 3) was prepared from ricinoleic acid by reduction of the Δ^9-double bond with diimide (generated from hydrazine in the presence of a trace of copper ion (18)). The absolute configuration of this acid has been conclusively established as D by Serck-Hanssen (19). The methyl ester (m.p. 56.5–57.0°; prepared by treatment of the acid with diazomethane and purified by silicic acid column chromatography and recrystallization from acetone-water) was found to be levorotatory in methanol ($[\alpha]_{546}$ −0.39 ± 0.03°; c = 2.19).

types of stearic acid molecules are converted to oleic acid at the same rate; *i.e.* there is no isotope effect on removal of the 9-D hydrogen.

Stearic acid labeled stereospecifically with deuterium was prepared and converted to oleic acid by the bacterial system as described below.

Stereospecifically Labeled 9-$^2H_1$-10-$^2H_1$-Stearic Acid-1-^{14}C—To a mixture of oleic acid-1-^{14}C (1.55 mg, 500 μc, New England Nuclear Corporation) and oleic acid (517 mg, Hormel Institute, Austin, Minnesota) in deuterated methanol (CH_3O^2H, 15 ml, Volk Radiochemical Company) was added deuterated hydrazine hydrate $N_2{}^2H_4 \cdot {}^2H_2O$, 6 g, Volk Radiochemical Company). Cupric acetate (1.5 mg) was added, and the reaction mixture was stirred at room temperature for 6½ hours in an atmosphere of dry air. The reaction mixture was poured into a mixture of dilute HCl and ether. The pH of the aqueous phase was 1. The ether layer was separated, and the aqueous phase was extracted once more with ether. The combined ether extracts were washed twice with water and dried over anhydrous sodium sulfate. The methyl esters, prepared by treatment of the acids with diazomethane, were heated under reflux with 4 g of mercuric acetate in 40 ml of methanol for 4 hours. The methanol was evaporated and the residue was applied to an activated silicic acid column (2 × 12 cm). The saturated ester (110 mg) was eluted with 5% ether in pentane. After saponification in methanolic KOH and acidification to pH 1 with dilute HCl, the acid was partitioned into ether. The ether extract was washed four times with water and dried over anhydrous sodium sulfate. Evaporation of the solvent and recrystallization from acetone-water yielded 9-$^2H_1$-10-$^2H_1$-stearic acid-1-^{14}C (90 mg, m.p. 68–69°). The purity, as judged by vapor phase chromatographic analysis of the methyl ester, was greater than 99%. The infrared spectrum was very similar to that of a sample of undeuterated stearic acid with the important exception that it showed strong C—D absorption at approximately 4.70 μ. Mass spectrometric analysis of the methyl ester indicated the following percentage composition: undeuterated (mol. wt. 298.5), 2.9; monodeuterated (mol. wt. 299.5), 6.3; and dideuterated (mol. wt. 300.5), 90.5.

TABLE IX

Microbiological desaturation of stereospecifically labeled 9-$^2H_1$-10-$^2H_1$-stearic acid-1-^{14}C

	Oleic acid molecules containing		
	No ^2H atoms	1 ^2H atom	2 ^2H atoms
	%	%	%
Expected* for			
Case 1, 10-D hydrogen removal	68	2	29
Case 2, 10-L hydrogen removal	39	61	0
Found	52	8	40

* Expected results are corrected for dilution by endogenous oleic acid and for the fact that the substrate was not fully dideuterated. The expected results were calculated as follows. The specific activity of the labeled substrate was 4.05 × 10^4 c.p.m. per μmole, and that of the methyl oleate recovered after incubation was 2.63 × 10^4 c.p.m. per μmole. From these changes in specific activity it can be calculated that 65% (2.63 × 10^4/4.05 × 10^4 × 100) of the methyl oleate was derived from the added substrate and 35% from endogenous origin. Therefore, 35% of the methyl oleate molecules recovered after the incubation will be undeuterated because they were not formed from the deuterium-labeled stearate. The deuterium-labeled substrate (which contributed 65% of the recovered oleate molecules) was not fully dideuterated, and corrections for this must be made. Of the stearate molecules, 2.9% contained no deuterium. These undeuterated molecules will account for 1.9% (2.9 × 0.65) of the total oleate molecules recovered from the incubation. Of the substrate molecules, 6.3% were monodeuterated and will contribute 4.1% (6.3 × 0.65) of the oleate molecules recovered after incubation. Since it can safely be assumed that the deuterium was distributed equally between the four possible positions (9-D, 9-L, 10-D, and 10-L), half of these monodeuterostearic acids will lose deuterium on conversion to oleate whether the 10-D or the 10-L hydrogen is removed. Therefore, the monodeuterated substrate molecules will contribute 2.05% (4.1 × 0.5) of the total oleate molecules recovered as monodeuterated oleate and 2.05% (4.1 × 0.5) as undeuterated oleate. Of the stearic acid substrate molecules, 90.5% were dideuterated and will contribute 58.8% (90.5 × 0.65) of the recovered oleate molecules. If the desaturation reaction involves stereospecific removal of the 10-D hydrogen, these substrate molecules will contribute, as dideuterated species, 29.4% (58.8 × 0.5) of the total oleate recovered and 29.4% (58.8 × 0.5) as undeuterated molecules. However, if the desaturation reaction involves stereospecific removal of the 10-L hydrogen, 58.8% of the oleate molecules, in the form of monodeuterated species, would be derived from the dideuterated substrate molecules. Totaling these percentages for the two cases, for stereospecific removal of either the 10-D or the 10-L hydrogen, yields the data shown in Table X.

TABLE X

Contribution from	Oleate molecules containing					
	with removal of 10-D hydrogen			with removal of 10-L hydrogen		
	No ^2H atoms	1 ^2H atom	2 ^2H atoms	No ^2H atoms	1 ^2H atom	2 ^2H atoms
	%	%	%	%	%	%
Endogenous fatty acid molecules	35	0	0	35	0	0
Substrate molecules containing						
No ^2H atoms	1.9	0	0	1.9	0	0
1 ^2H atom	2.05	2.05	0	2.05	2.05	0
2 ^2H atoms	29.4	0	29.4	0	58.8	0
Total	68	2	29	39	61	0

Microbiological Desaturation of Stereospecifically Labeled 9-$^2H_1$-10-$^2H_1$-Stearic Acid-1-^{14}C—9-$^2H_1$-10-$^2H_1$-Stearic acid-1-^{14}C (7.706 mg, 1.09 × 10^6 c.p.m.) in 8 ml of ethanol was added to 1000 ml of culture medium which had been inoculated with *C. diphtheriae*. The incubation was carried out for 28 hours at 30° on a rotating shaker. The bacteria were isolated, washed, and weighed (9.4 g). The total cellular fatty acid methyl esters were isolated and separated into saturated (4.04 × 10^5 c.p.m.) and unsaturated (3.61 × 10^5 c.p.m.) ester fractions as described above. The unsaturated esters were subjected to vapor phase chromatography, and methyl oleate was collected at the exit of the column, weighed (2.71 mg), and assayed for radioactivity (2.404 × 10^5 c.p.m.).

The results of the mass spectrometric analysis of the methyl oleate are given in Table IX, together with the results expected for removal of either the 10-D or the 10-L hydrogen. In calcu-

lating the expected results, corrections were made for endogenous dilution and for the fact that the substrate was not fully dideuterated. The finding that 40% of the methyl oleate molecules were dideuterated leads to the conclusion that the hydrogen removed from carbon atom 10 of stearic acid on enzymatic conversion to oleic acid has the D configuration. If the desaturation reaction involved stereospecific removal of the 10-L hydrogen, no dideuterated oleate molecules would be formed.

The formation of 8% monodeuterated molecules, as compared with the expected 2%, is most likely the result of a small amount of *trans* addition of deuterium during the chemical synthesis of the dideuterated stearic acid. The fact that the percentage of dideuterated oleic acid molecules was somewhat higher than expected (40 as compared to 29) is compatible with isotope discrimination in the course of the desaturation reaction. The results obtained with the tritium-labeled substrate had indicated the presence of an isotope effect during the enzymatic removal of the 9-D hydrogen, but not on removal of the hydrogen from carbon atom 10. It is therefore to be expected that, in the experiment with the deuterium-labeled substrate, those molecules containing hydrogen in the 9-D configuration—and hence deuterium in the 9-L configuration—will undergo desaturation at a faster rate than the molecules containing deuterium in the 9-D configuration. The experimental results are consistent with these considerations.

From the experiments described above it is possible to deduce the absolute configuration of the optically active 10-hydroxystearic acid that is formed by the action of the pseudomonad on oleic acid. The results obtained with the dideuteriostearic acid establish that the hydrogen removed from carbon atom 10 of stearic acid during the enzymatic conversion to oleic acid is of the D configuration. The 10-^{3}H-stearic acid from which tritium was removed upon enzymatic conversion to oleic acid originated from the tosylate of the dextrorotatory 10-hydroxystearate. Since the tritium in the stearic acid was of the D configuration and since it was introduced by a reaction involving an inversion of configuration, it follows that the dextrorotatory 10-hydroxystearate was of the L configuration. The levorotatory 10-hydroxystearate produced by the pseudomonad from oleic acid is therefore the D enantiomorph.

DISCUSSION

The purpose of this investigation was to determine whether the conversion of stearic acid to oleic acid in *C. diphtheriae* is stereospecific with respect to the hydrogens removed from carbon atoms 9 and 10. The experimental finding that only one of each of the pair of hydrogens at carbon atoms 9 and 10 is removed shows that the action of the desaturating enzyme is indeed highly stereospecific. The absolute configuration of both of these hydrogens is D. Despite the fact that the absolute configurations of the hydrogens removed from carbon atoms 9 and 10 are both D, it does not necessarily follow that the desaturation reaction involves "*cis*" removal of the 2 hydrogens. This restriction arises from the fact that the experiments establish only the configurations of the hydrogens removed from the stearic acid and the configuration of the resulting olefin. Neither the precise mechanism of the desaturation reaction nor the conformation of the substrate molecule at the active site of the enzyme is known. If the two D hydrogens that are removed assume an eclipsed conformation in the enzyme-substrate complex, then the desaturation reaction would indeed proceed by *cis* elimination of the two hydrogens. However, it is also possible that the reaction involves stereospecific removal of 2 "*trans*" hydrogens. For this case the 2 leaving hydrogens would have to assume the staggered conformation at the active site of the enzyme. Removal of 2 *trans* hydrogens to yield a *cis*-olefin could arise either by a stereospecific change in conformation, from staggered to eclipsed, of an enzyme-bound intermediate in the desaturation reaction or by an isomerization of an enzyme-bound *trans*-olefin to the *cis*-olefinic oleic acid. Enzymes capable of isomerizing elaidic acid (*trans*) to oleic acid (*cis*) are not known. Nevertheless, a change in configuration of an enzyme-bound olefinic intermediate during the course of the over-all desaturation reaction cannot be excluded.

The findings suggesting the presence of an isotope effect on removal of the hydrogen from carbon atom 9, but not on removal of the hydrogen from carbon atom 10, have important mechanistic implications. They suggest that the removal of the 2 hydrogens is not synchronous but that removal of hydrogen from carbon atom 9 precedes removal of hydrogen from carbon atom 10. Studies are in progress to confirm the presence of this isotope effect and to ascertain its magnitude.

SUMMARY

Four stereospecifically labeled monotritiostearic acids were prepared from the enantiomorphic pairs of 9-hydroxyoctadecanoate and 10-hydroxyoctadecanoate. The four labeled stearic acids were incubated with growing cultures of a strain of *Corynebacterium diphtheriae*, and the oleic acid produced by the organism was isolated in each case. Loss of tritium occurred with the 9-D- and 10-D-tritiostearic acids, but not with the two L-tritio compounds. The conversion of stearate to oleate is therefore stereospecific with respect to the removal of hydrogen at carbon atoms 9 and 10. Isotope effects observed in the formation of oleate suggest that hydrogen removal at carbon atom 9 precedes hydrogen removal at carbon atom 10 of stearate.

Acknowledgments—The authors wish to express their sincere gratitude to Mr. J. Occolowitz and Professor K. Biemann for the mass spectral analyses of the deuterated compounds.

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George J. Schroepfer, Jr. and Konrad Bloch

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