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Stereospecific Conversion of Oleic Acid to 10-Hydroxystearic Acid*

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SUMMARY

The conversion of oleic acid to 10-D-hydroxystearic acid by a pseudomonad was studied in a medium enriched in deuterium oxide. The reaction occurred with stereospecific incorporation of 1 atom of solvent hydrogen at carbon atom 9. The location of the deuterium was established by a combination of chemical and mass spectrometric evidence. The hydroxyl function of the deuterium-labeled hydroxystearate was replaced by hydrogen through a series of standard organic reactions which should not affect the stereochemistry at carbon atom 9. The resulting deuterium-labeled stearate was incubated with a growing culture of a strain of *Corynebacterium diphtheriae*, a system which stereospecifically removes hydrogen in the D configuration at carbon atom 9 of stearate in the enzymatic introduction of the Δ^9-double bond of oleic acid. Retention of the labeled hydrogen in the oleate was observed, indicating that the configuration of the deuterium in the stearate, and hence in the 10-hydroxystearate, was L.

The microbiological conversion of oleic acid to 10-hydroxystearic acid is characterized by notable stereospecificity. The 10-hydroxystearate formed in this reaction is optically active (1, 2) and has the D configuration (2). The purposes of the present study were to determine the number of atoms of solvent hydrogen taken up during the course of this reaction and to investigate the possible stereospecific nature of this process. A preliminary account of some of this work has been published (3).

EXPERIMENTAL PROCEDURE AND RESULTS

General Procedures—Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. Radioactivity was measured in a Packard liquid scintillation spectrometer. [1-^{14}C]-Stearic acid was purchased from the New England Nuclear Corporation, and it was purified by mercuric acetate treatment and silicic acid column chromatography. Methyl esters were prepared by treatment of the acids with diazomethane (4). Potassium borodeuteride and lithium aluminum deuteride were purchased from Metal Hydrides. Infrared spectra were recorded on a Beckman IR-5A spectrometer with KBr micropellets. Mass spectrometric analyses were carried out on an Atlas CH4 mass spectrometer with a direct sample inlet system. Multiple measurements (an average of six for each reference and deuterated compound) were made at an ionizing voltage of 70 e.v. Spectra obtained at lower ionizing voltages (14 to 25 e.v.) were also studied so as to avoid any errors in the determination of deuterium content because of isotope effects in the fragmentation processes. Calculations of isotopic composition were made according to Biemann (5).

Microbiological Formation of 10-D-Hydroxy-[9-$^2H_1$]-stearic Acid from Oleic Acid—The pseudomonad (NRRL-2994)[1] was incubated with oleic acid in the presence of deuterium oxide. Tryptone from Difco (2.0 g), K_2HPO_4 (0.4 g), and glucose (0.4 g) were dissolved in deuterium oxide (380 ml; 99.8% ^2H), and the pD of the resulting solution was adjusted to 8.4 by the addition of KO^2H (two KOH pellets dissolved in 7.5 ml of ^2H_2O). After autoclaving for 20 min, a suspension of the bacteria (1 ml, nondeuterated medium) was added. The incubation was carried out for 24 hours at 24° on a rotatory shaker. Oleic acid from Mann (5.025 g, diene content <0.03%, iodine number 89) was added to the culture, and the incubation was continued for 84 hours. The bacteria were sedimented by centrifugation, and the culture liquors were acidified to pH 1 by the addition of cold 6 N H_2SO_4. The fatty acids were extracted with ether, and the ether solution was washed twice with water and dried over anhydrous magnesium sulfate. After evaporation of the solvent and drying in a vacuum, the fatty acids (3.29 g) were applied to a silicic acid (150 g from Mallinckrodt)-Super Cel (45 g, from Johns-Manville) column, and the nonpolar acids were eluted with 10% ether in Skellysolve B. The polar acids were eluted with 50% ether in Skellysolve B, and, after methylation with diazomethane, the methyl esters were subjected to chromatography on an activated silicic acid columm. The fraction, eluted with 25% ether in pentane, yielded after evaporation of the solvent a white solid (935 mg) which was recrystallized from methanol. The crystals of the first crop (701 mg) melted at 55.5–56.0°. The compound was indistinguishable from undeuterated methyl 10-hydroxystearate upon vapor phase chromatographic analysis. A single spot was observed on thin layer chromatographic analysis. The

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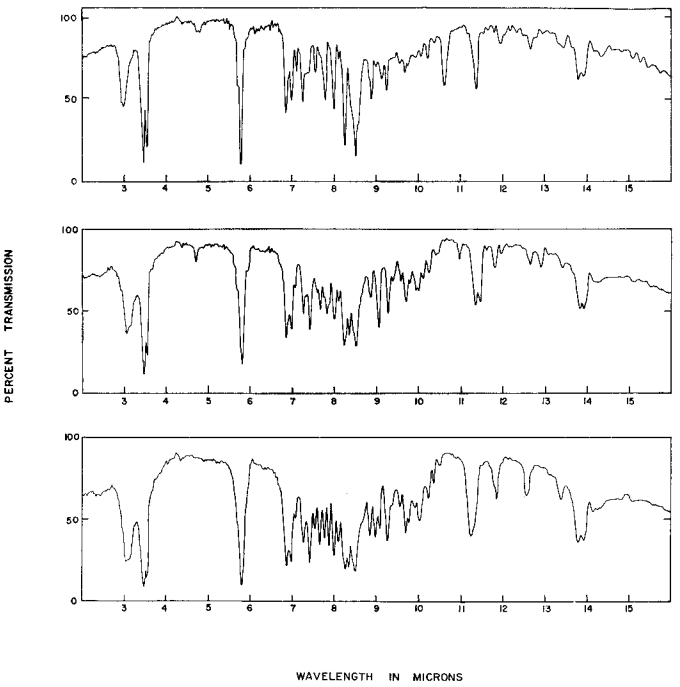

FIG. 1. Infrared spectra of methyl 10-hydroxystearates. *Bottom*, methyl 10-D-hydroxystearate; *middle*, methyl 10-D-hydroxy-[9-L-$^2H_1$]-stearate; *top*, methyl 10-DL-hydroxy-[10-DL-$^2H_1$]-stearate.

presence of deuterium in the compound was indicated by the absorption at 4.70 μ (C—D stretch) in the infrared spectra of the methyl ester (Fig. 1) and the free acid. The fingerprint region of the spectra differed significantly from that of the spectra of the corresponding undeuterated compounds as has been reported previously in several cases of deuterium-labeled compounds (6–10). Apart from the absorption at 4.70 μ, the following differences were noted in the spectra of the deuterated and nondeuterated methyl esters. The absorption at 7.58 μ in the reference compound was markedly reduced in intensity in the case of the deuterated molecule; the absorption at 7.78 μ was replaced, in the case of the deuterated compound, by a peak at 7.81 μ with shoulders at 7.75 μ and 7.86 μ; the addition of a shoulder at 8.44 μ on the absorption peak at 8.50 μ; the absence of bands at 8.99 μ and 9.10 μ and the presence of new bands at 9.05 μ, 10.10 μ, 10.97 μ, 11.60 μ, 11.97 μ, 12.90 μ; a reduction in the intensity of the absorption at 10.02 μ; and replacement of the broad, intense absorption at 11.25 μ in the undeuterated compound by a doublet with peaks at 11.35 μ and 11.48 μ.

Analysis of the methyl ester by mass spectrometry (Fig. 2) indicated a molecular weight of 315 corresponding to the incorporation of 1 deuterium atom. Analysis of the precise deuterium content in the molecule was difficult owing to the low intensity of the molecular ion (M) in methyl 10-hydroxystearate and the presence of a significant peak of variable intensity at M − 1. Calculation of the deuterium content of the compound was carried out on the fragment *m/e* 283 (M − 31) corresponding to the loss of OCH_3. This peak was shifted to *m/e* 284 in the deuterated compound. The isotopic composition of the compound is shown in Table I.

The results indicate the uptake of 1 atom of stably bound deuterium during the course of the enzymatic reaction. That most, if not all, of the deuterium was located on carbon atom 9 was indicated by chemical and mass spectrometric studies which are described below in this paper.

[9-$^2H_1$]Stearyl Alcohol from 10-Hydroxy-[9-$^2H_1$]-stearic Acid—To methyl 10-hydroxy-[9-$^2H_1$]-stearate (559 mg) in dry pyridine (40 ml, cooled to −10°) was added *p*-toluenesulfonyl chloride (4.5 g). The solution was kept at −10° for 11 days. Cold water (70 ml) was added, and the resulting mixture was extracted three times with ether. The combined ether solutions were washed seven times with water and dried over anhydrous magnesium sulfate. Evaporation of the solvent yielded a viscous oil (779 mg) which was applied to an activated silicic acid column (50 g) in 5% ether in pentane. After passing 480 ml of the same solvent mixture through the column, the tosylate (751 mg) was recovered by elution with 15% ether in pentane (480 ml). The methyl 10-tosyl-[9-$^2H_1$]-stearate (571 mg) was dissolved in anhydrous ether and heated under reflux with lithium aluminum hydride (2.435 g) for 54 hours. After decomposing the excess hydride by the successive additions of ethyl acetate and water, the mixture was acidified to pH 1 with dilute HCl and it was extracted three times with ether. The combined ether solutions were washed with water and dried over anhydrous magnesium sulfate. The solvent was evaporated, and the residue (447 mg) was applied to a 2:1 silicic acid-Super Cel column (100 × 2 cm) in benzene, and the column was eluted with benzene. Fractions, 11 ml in volume, were collected by means of an automatic fraction collector. The contents of Fractions 125 to 170 were pooled and recrystallized from acetone-water, yielding [9-$^2H_1$]-octadecanol-1 (157 mg, m.p. 57.5–58.0°). The infrared spectrum was very similar to that of an undeuterated sample of 1-octadecanol with the exception of the presence of an absorption band at 4.70 μ (C—D stretch) and the absorption in the 15.0 to 15.4 μ region of the spectrum of the deuterated sample. In addition, there appear to be some differences in the series of bands between 7.4 μ and 8.5 μ (associated with rocking or twisting motions of the methylene groups in a polymethylene chain) although these peaks are very weak in the spectrum of octadecanol.

Vapor phase chromatographic analysis indicated that the purity was in excess of 99%. The deuterated octadecanol had the same retention time as that of an undeuterated sample. Analysis of the deuterated 1-octadecanol by mass spectrometry indicated a molecular weight of 271 corresponding to the incorporation of 1 deuterium atom. Determination of the precise deuterium content by analysis of the molecular ion peak was not possible because of its low intensity and the presence of a sizeable M − 1 peak. At lower ionizing voltages (14 to 16 e.v.) the relative intensity of the molecular ion peak remained low and a sizeable M − 1 peak persisted. Because of these considerations analysis of the deuterium content was based upon the fragment M − 18, a prominent peak in the spectrum of 1-octadecanol, which corresponds to the loss of water. The fragmentation of 1-butanol has been investigated by McFadden *et al.* (11, 12) and MacDonald, Shannon, and Sugowdz (13) who found, by deuterium labeling, that the dehydration results mainly from 1,3- and 1,4-eliminations. The validity of the use of the M − 18 fragment for calculation of the deuterium content of the molecule is therefore dependent on the absence of deuterium from carbon atoms 3 and 4. Evidence indicating that the deuterium is not located on carbon atoms 3 and 4 is presented in a later section. The isotopic composition, calculated on the basis of this fragment, is listed in Table I.

[9-$^2H_1$]-Stearic Acid from [9-$^2H_1$]-Stearyl Alcohol—To [9-$^2H_1$]-

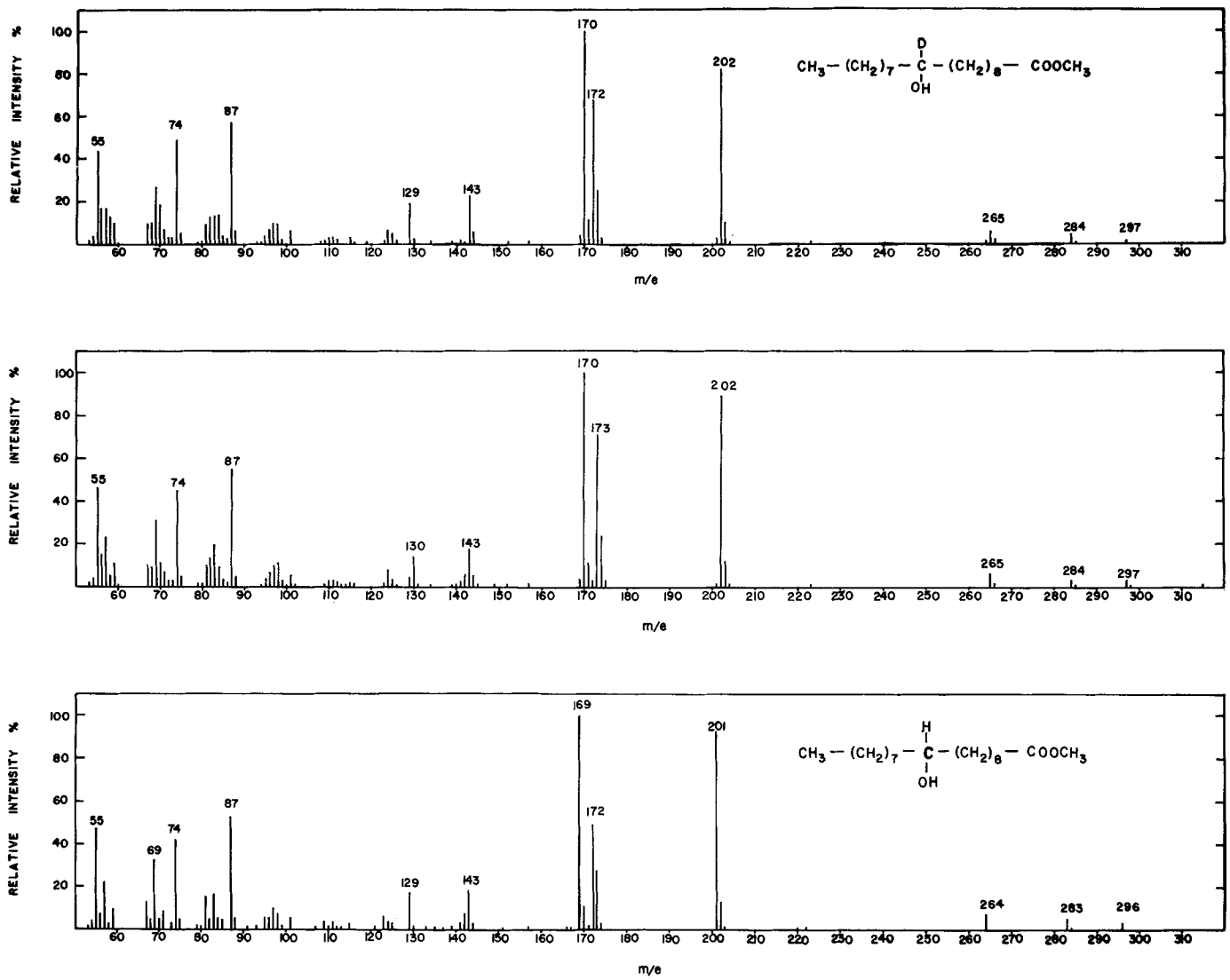

FIG. 2. Mass spectra of methyl 10-hydroxystearates. *Bottom*, methyl 10-D-hydroxystearate; *middle*, methyl 10-D-hydroxy-[9-L-$^2H_1$] stearate; *top*, methyl 10-DL-hydroxy-[10-DL-$^2H_1$]-stearate. Ionizing voltage was 70 e.v. All peaks in the range *m/e* 50 to 320 which were at least 1% of the intensity of the base peak have been plotted.

stearyl alcohol (110 mg) in glacial acetic acid (12.4 ml) was added chromium trioxide (151 mg) in 90% acetic acid (15 ml). After standing 24 hours at room temperature, water was added, and the crude stearic acid was collected on a scintered glass filter and washed with water. The crude acid was heated on a steam bath with aqueous methanolic KOH and washed with petroleum ether to remove any unreacted octadecanol. After acidification to pH 1, the acid was extracted with ether, and the ether solution was washed with water and dried over anhydrous magnesium sulfate. Evaporation of the solvent and recrystallization from acetone yielded [9-$^2H_1$]-stearic acid (75 mg, m.p. 68–69°). The infrared spectrum was similar to that of an undeuterated sample of stearic acid (Fig. 3). In addition to the absorption band at 4.70 μ (C—D stretch), there are several other differences in the spectra of the monodeuterated and the unlabeled stearates. Also shown in Fig. 3 is the spectrum of a sample of [9-$^2H_1$]-[10-$^2H_1$]-stearic acid, prepared previously by chemical synthesis (2). The spectra of the monodeuterated and dideuterated samples differ significantly from the undeuterated stearate in the region from 7.4 to 8.5 μ, a region containing a series of bands associated with rocking or twisting motions of the methylene groups in a polymethylene chain. The differences in this region, chiefly between 8.0 μ and 8.5 μ in the case of the monodeuterated compound, are much more marked in the case of the dideuterated compound. The band at 13.20 μ in the spectrum of stearic acid is shifted slightly (to 13.38 μ) in the spectra of the mono- and dideuterated stearates.

Vapor phase chromatographic analysis of the methyl ester of the monodeuterated acid indicated that the purity was in excess of 99%. The retention times of the deuterated and undeuterated methyl stearates were the same under the conditions employed. Analysis of the deuterated methyl stearate by mass spectrometry indicated a molecular weight of 299 corresponding to the incorporation of 1 deuterium atom. The molecular ion of methyl stearate is of considerable intensity and is not accompanied by a sizeable M − 1 peak. Analysis of the deuterium content, based on the molecular ion, indicated the isotopic composition shown in Table I.

Evidence for Localization of Labeled Hydrogen at Carbon Atom 9 of 10-Hydroxystearic Acid—Assignment of the location of the

TABLE I

Isotopic composition of deuterium-labeled compounds

Sample	Molecules ± S.E.		
	Undeuterated	Monodeuterated	Dideuterated
	%	%	%
1. Methyl 10-D-hydroxystearate derived from incubation of oleate with pseudomonad in D_2O.....	1.7 ± 0.2	97.0 ± 0.5	1.6 ± 0.4
2. Stearyl alcohol prepared from Sample 1.....	2.4 ± 0.5	97.1 ± 0.9	0 ± 0.4
3. Methyl stearate prepared from Sample 2.....	1.6 ± 0.2	98.4 ± 0.8	0 ± 0.3
4. Methyl 10-ketostearate prepared from Sample 1 and treated with base.	100 ± 1.1	0 ± 0.6	0 ± 0.3
5. Methyl oleate obtained from incubation of stearate with *C. diphtheriae*	24.8 ± 1.6	75.2 ± 0.5	0 ± 1.5

deuterium atom at carbon atom 9 of the 10-hydroxystearate was based on a combination of chemical and mass spectrometric studies. The methyl ester of the deuterated 10-hydroxystearic acid was subjected to chromic acid oxidation under the conditions described by Ross, Gebhardt, and Gerecht (14). The crude 10-keto ester (m.p. 41–42°) was heated under reflux for 2 hours with KOH (20 g) in 80% methanol (100 ml) and allowed to stand overnight at room temperature. The free acid was isolated in the usual manner and recrystallized from acetone-water and pentane (m.p. 81.0–81.5°). No C—D absorption was present in the infrared spectra of concentrated preparations of this compound. The methyl ester was prepared by treatment with diazomethane, and it was purified by recrystallization from heptane (m.p. 44°). The purity, as judged by thin layer and gas-liquid chromatographic analyses, was in excess of 99%. No C—D absorption was observed in the infrared spectra of this compound. An authentic sample of methyl 10-ketostearate (m.p. 46–47°) was prepared as described above from methyl 10-D-hydroxystearic acid. Its purity was in excess of 99% as judged by thin layer and gas-liquid chromatographic analyses. The mass spectra obtained from the authentic 10-ketostearate and from the 10-ketostearate prepared from the deuterated 10-hydroxystearate were essentially the same, indicating the absence of labeled hydrogen. Precise calculation of the deuterium content (based on the molecular ion) indicated the composition shown in Table I.

The loss of deuterium upon oxidation of the hydroxystearate and treatment of the resulting ketone with base localizes the deuterium to carbon atoms 9, 10, and 11. Labeled hydrogen at carbon atom 2 of the hydroxystearate would also be removed by the alkali treatment, but the possibility of carbon atom 2 as the location of the deuterium atom can be excluded on the basis of the stability of the deuterium in the 10-hydroxystearate to the acidic conditions used in the isolation of this compound and, more definitively, on the basis of the stability of the labeled hydrogen in the stearic acid (prepared from the deuterated 10-hydroxystearic acid) to treatment with alkali.

Valuable information concerning the location of the deuterium in the hydroxystearate was obtained by analysis of the mass spectra of the deuterated 10-hydroxystearate (Fig. 2). A major peak in the spectrum of methyl 10-hydroxystearic acid is at *m/e* 201, representing the fragment $HOCH\text{-}(CH_2)_8\text{-}COOCH_3$ (15). In the spectrum of the deuterated compound this peak occurs at *m/e* 202, indicating that the deuterium is not on carbon atom 11. The base peak in the spectrum of the authentic 10-hydroxystearate is at *m/e* 169, corresponding to the fragment mentioned above (*m/e* 201) minus 32. In the spectrum of the deuterated hydroxystearate this peak is shifted to *m/e* 170. Another prominent peak in the spectrum of methyl 10-hydroxystearate is at *m/e* 172 which has been assigned to the fragment $(CH_2)_8\text{-}COOCH_3$ plus a hydrogen atom (15). In the spectrum of the deuterated compound this peak occurs at *m/e* 173, indicating that the deuterium is probably not on carbon atom 10. However, since the origin of the "extra" hydrogen atom in this fragment has not been established, there remains the remote possibility that the deuterium in the 10-hydroxystearate was located on carbon atom 10 and that the extra hydrogen in this fragment had its exclusive origin from this position.

Several lines of evidence can be cited which are inconsistent with this possibility. Methyl 10-DL-hydroxy-[10-DL-$^2H_1$]-stearic

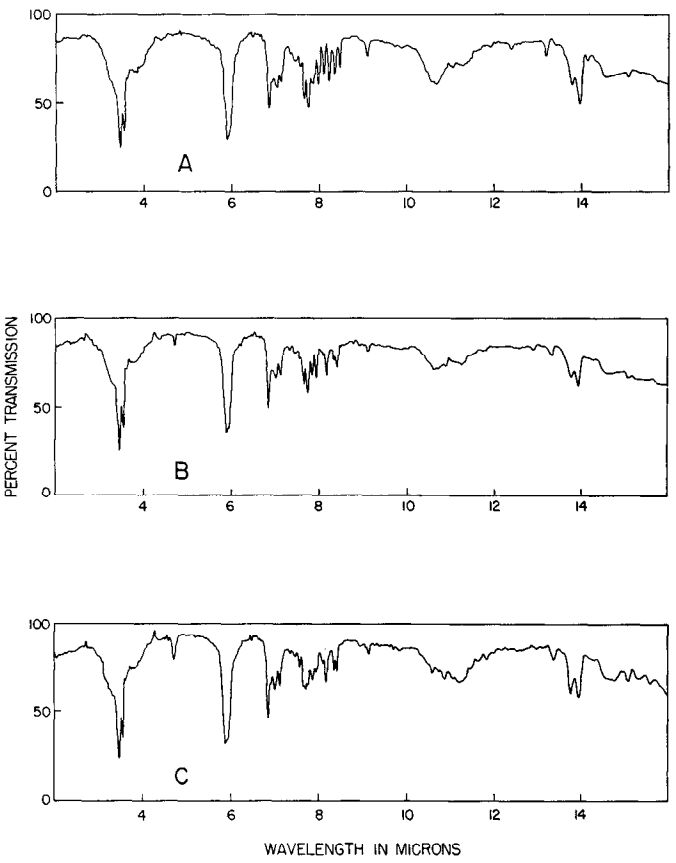

FIG. 3. Infrared spectra of stearic acids. *A*, stearic acid; *B*, [9-L-$^2H_1$]-stearic acid; *C*, [9-DL-$^2H_1$]-[10-DL-$^2H_1$]-stearic acid.

acid was prepared from methyl 10-ketostearic acid by reduction with sodium borodeuteride. The product was recrystallized from acetone-water (m.p. 50–51°, unchanged by further recrystallization from methanol; purity in excess of 99% as judged by thin layer and gas-liquid chromatography) and analyzed by mass spectrometry (Fig. 2). The peaks at *m/e* 201 and *m/e* 169 in the spectrum of the unlabeled hydroxystearate representing $HOCH\text{-}(CH_2)_8\text{-}COOCH_3$ and this fragment minus 32, respectively, are shifted up 1 mass unit in the spectrum of the [10-$^2H_1$]-compound. However, the peak at *m/e* 172 in the unlabeled hydroxystearate, representing the fragment $(CH_2)_8\text{-}COOCH_3$ plus a hydrogen atom, is not shifted in the spectrum of the 10-deuterated compound. Therefore, the extra hydrogen in this fragment does not have its origin from carbon atom 10, and the deuterium in the enzymatically formed 10-hydroxystearate is not on carbon atom 10.

The peak at *m/e* 129 in the spectrum of methyl 10-hydroxystearate is relatively intense. That this peak largely represents the fragment ($CH_3OOC\text{-}(CH_2)_8$ plus a hydrogen atom) minus 43 is suggested by the observation that similar relatively intense peaks occur at *m/e* 101 ($CH_3OOC\text{-}(CH_2)_6$ + hydrogen minus 43) in the spectrum of methyl 8-hydroxystearate and at *m/e* 227 ($CH_3OOC\text{-}(CH_2)_{15}$ + hydrogen minus 43) in the spectrum of methyl 17-hydroxystearate (15). The 43 mass units lost may have their origin from carbon atoms 2, 3, and 4 plus a hydrogen atom as has been suggested for the commonly observed peaks at *m/e* = M − 43 in the spectrum of fatty acid methyl esters (16). The finding that the peak at *m/e* 129 in the spectrum of methyl 10-hydroxystearate is, for the most part, shifted to *m/e* 130 in the spectrum of methyl 10-hydroxy-[9-$^2H_1$]-stearate (but not in the case of methyl 10-DL-hydroxy-[10-DL-$^2H_1$]-stearate) is compatible with these suggestions. In addition, a metastable peak was observed at *m/e* ~ 96.8 (calculated: $(129)^2/172$, or 96.75) in the spectrum of methyl 10-hydroxystearate. Moreover, a metastable peak at *m/e* ~ 97.8 (calculated $(130)^2/173$, or 97.7) was observed in the spectrum of the methyl 10-hydroxy-[9-$^2H_1$]-stearate.

The infrared spectrum of the 10-monodeuterated 10-hydroxystearate also differed significantly from that of the 9-monodeuterated 10-hydroxystearate and that of unlabeled 10-hydroxystearate (Fig. 1). The C—D stretch appears as a doublet with small peaks at 4.70 μ and 4.80 μ. Similar splitting of the absorption caused by the C—D stretch in related acyclic compounds has been reported by Frejaville *et al.* (10). For example, 3-monodeuterio-3-hydroxypentane showed absorption at 4.66 μ and 4.78 μ. Bands seen in 10-hydroxystearate which are absent or markedly reduced in the spectrum of the 10-monodeuterated 10-hydroxystearate are those at 7.40 μ, 7.65 μ, 7.88 μ, 8.10 μ, 8.35 μ, 8.99 μ, and 10.50 μ. In addition, the deuterated compound shows an intense new band at 10.62 μ and several smaller bands at 12.16 μ, 12.31 μ, 13.30 μ, 15.10 μ, and 15.67 μ. Three peaks are found at 11.65 μ, 11.80 μ, and 11.92 μ in the place of the single peak at 11.86 μ in the undeuterated compound.

The results obtained in the studies of the microbiological conversion of the monodeuterated stearic acid to oleic acid (see below) are also incompatible with location of the deuterium on carbon atom 10. This statement arises from the following considerations. Deuterium at carbon atom 10 of 10-D-hydroxystearate would have to be in the L configuration. Hydrogenolysis of the tosylate of 10-D-hydroxy-[10-L-$^2H_1$]-stearate with lithium aluminum hydride would yield [10-D-$^2H_1$]-stearic acid (after oxidation of the resulting octadecanol). Since the enzymatic desaturation reaction involves stereospecific removal of the 10-D-hydrogen (2), the virtually complete retention of deuterium observed on conversion of stearate to oleate (see below) is incompatible with location of the deuterium in the D configuration of stearate (or the L configuration of 10-D-hydroxystearate).

In an attempt to provide further evidence for the localization of the deuterium at carbon atom 9, the mass spectra of the monodeuterated stearyl alcohol and methyl stearate prepared from the enzymatically formed monodeuterated 10-hydroxystearate have been studied. For reference compounds, [9-L-$^2H_1$]-stearyl alcohol, [10-L-$^2H_1$]-stearyl alcohol, methyl [9-L-$^2H_1$]-stearate, and methyl [10-L-$^2H_1$]-stearate were prepared by chemical synthesis. [1,1,9-L-$^2H_3$]-Stearyl alcohol and [1,1,10-L-$^2H_3$]-stearyl alcohol were prepared from the tosylates of methyl 9-D-hydroxystearate and methyl 10-D-hydroxystearate by reduction with lithium aluminum deuteride. The resulting trideuterated octadecanols were converted to their corresponding acids by chromic acid oxidation. [9-L-$^2H_1$]-Stearyl alcohol and [10-L-$^2H_1$]-stearyl alcohol were prepared from the corresponding monodeuterated acids by reduction with lithium aluminum hydride. The purity of the stearyl alcohols and stearates was judged to be in excess of 99% as judged by gas-liquid chromatography. The mass spectra of the three deuterated alcohols were found to be essentially indistinguishable. This is compatible with the proposed fragmentation processes involving predominantly olefinic ions (17).

In a preliminary report (3), the apparent lack of shift of the peak at *m/e* 127 of methyl stearate to *m/e* 128 in the spectrum of the monodeuterated stearate prepared from the enzymatically formed [$^2H_1$]-10-hydroxystearate was cited as evidence for the absence of deuterium at carbon atom 10. This peak, compatible with a hydrocarbon fragment C_9H_{19} and a significant peak in the spectrum of *n*-alkanes, is in fact a very insignificant peak in the spectrum of methyl stearate and should not have been used for the localization of the deuterium.

The spectrum of the [$^2H_1$]-stearate prepared from the enzymatically formed [$^2H_1$]-10-hydroxystearate does differ significantly from the spectrum of chemically synthesized [10-L-$^2H_1$]-stearate, thus providing further evidence against the location of the deuterium at carbon atom 10. The mass spectra of the [$^2H_1$]-stearate prepared from the enzymatically formed [$^2H_1$]-10-hydroxystearate and the chemically synthesized [9-L-$^2H_1$]-stearate are virtually identical. The 9- and 10-monodeuterated stearates differ in the following respects. The peak at *m/e* 171 in the spectrum of methyl stearate is shifted up 1 mass unit in the spectrum of methyl [9-L-$^2H_1$]-stearate but not in the spectrum of methyl [10-L-$^2H_1$]-stearate. The peak at *m/e* 199 in the spectrum of the unlabeled stearate is largely shifted to *m/e* 200 in the spectrum of the [9-L-$^2H_1$]-stearate. However, in the case of the [10-L-$^2H_1$] in stearate, significant peaks appear at both *m/e* 199 and *m/e* 200. The peak at *m/e* 213 of methyl stearate is accompanied, in the spectrum of [9-L-$^2H_1$]-stearate, by a peak at *m/e* 214 which is of significantly greater intensity than that of the normal isotope peak. In the spectrum of [10-L-$^2H_1$]-stearate this peak appears at *m/e* 214.

Determination of Absolute Configuration of Deuterium at Carbon Atom 9—[9-$^2H_1$]-Stearic acid (20.02 mg) and [1-^{14}C]-stearic acid (22.1 μg, 6.28×10^5 cpm) in ethanol (8 ml) were added to a

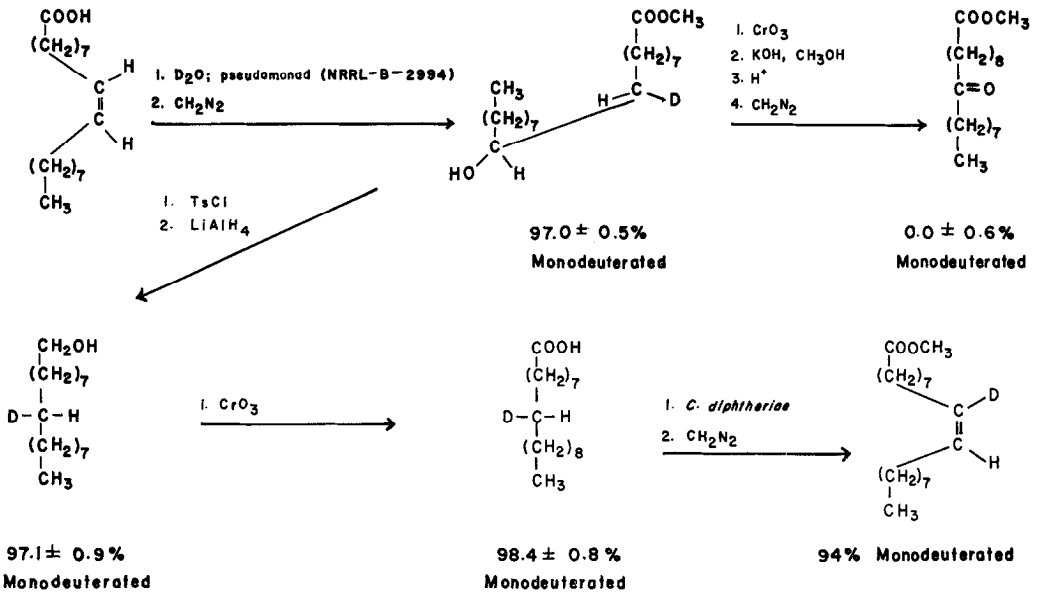

FIG. 4. Stereospecific nature of the incorporation of solvent hydrogen into 10-D-hydroxystearic acid

total of 3 liters of the culture medium of Mueller and Miller (18), supplemented with L-tryptophan (0.3 g), L-glutamic acid (1.5 g), and maltose (60 g). The medium was innoculated with a suspension of *Corynebacterium diphtheriae* strain $C_7S(—)$ tox — (19) kindly provided by Professor A. M. Pappenheimer. The bacteria were allowed to grow in the presence of the labeled acid for 48 hours at 31° on a rotatory shaker. The bacteria were isolated by centrifugation, washed with water, and weighed (27 g). The total cellular fatty acids were isolated and purified as the methyl esters as described previously (2). The total cellular fatty acid methyl esters (328 mg) were separated into saturated (2.63×10^5 cpm) and unsaturated (2.52×10^5 cpm) fatty acid methyl ester fractions as described previously (2). Methyl oleate (3.15 mg, 7.57×10^4 cpm) was isolated from the unsaturated ester fraction by preparative vapor phase chromatography and was further purified by silicic acid column chromatography. The purity, as judged by vapor phase chromatographic analysis, was in excess of 98%. Analysis of the methyl oleate by mass spectrometry (based on molecular ion peak) gave the isotopic composition shown in Table I.

The specific activity of the labeled substrate was 8.93×10^3 cpm per μmole, and that of the methyl oleate recovered after incubation was 7.11×10^3 cpm per μmole. From the change in specific activity observed in the substrate and product, it can be estimated that approximately 80% ($100 \times 7.11 \times 10^3/8.93 \times 10^3$) of the oleate molecules were derived from the added substrate. Correcting for this dilution ($75.2 \times 1/0.80$), approximately 94% of the oleate molecules derived from the incubated monodeuterated [9-$^2H_1$]-stearic acid contained 1 deuterium atom per molecule. This value is not corrected for the fact that the added substrate was not fully monodeuterated.

The enzyme system in this strain of *C. diphtheriae* responsible for the conversion of stearic acid to oleic acid has been shown to be stereospecific with respect to the hydrogen removal from carbon atom 9 of stearic acid. The hydrogen in the D configuration is specifically removed upon enzymatic formation of oleate from stearate. In the present study the [9-$^2H_1$]-stearic acid, derived from 10-D-hydroxy-[9-$^2H_1$]-stearic acid, was incubated with the *C. diphtheriae*. Virtually complete retention of the deuterium upon conversion of the added stearate to oleate indicates that most, if not all, of the deuterium at carbon atom 9 was in the L configuration. Since the reactions used to prepare the [9-L-$^2H_1$] stearic acid from the 10-hydroxy-[9-$^2H_1$]-stearate should not affect the stereochemistry at carbon atom 9, it follows that the deuterium at carbon atom 9 of the 10-hydroxystearate was also in the L configuration.

DISCUSSION

The microbiological conversion of oleic acid to 10-hydroxystearic acid provides a unique model for the study of the stereochemistry of enzymatic reactions at an isolated double bond in an acyclic compound. The results of the present study, summarized in Fig. 4, indicate that this reaction proceeds with stereospecific uptake of 1 atom of solvent hydrogen into the L configuration (*R* in the nomenclature system of Cahn, Ingold, and Prelog (20)) at carbon atom 9 of the 10-D-hydroxystearate (10*R* in the nomenclature system of Cahn, Ingold, and Prelog (20)).

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