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Stoichiometry and Stereochemistry of Deuterium Incorporated into Fatty Acids by Cells of *Escherichia coli* Grown on [*methyl*-$^2H_3$]Acetate†

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ABSTRACT: The incorporation of deuterium into the C_{12}, C_{14}, and C_{16} saturated fatty acids biosynthesized by *Escherichia coli* grown in a medium containing [*methyl*-$^2H_3$]acetate was studied. The incorporation of deuterium into these saturated fatty acids from three distinct populations of deuterium offers the best explanation for the observed isotopic distribution. The most enriched of these populations consisted of the terminal methyl group in which 80% of each fatty acid contained a methyl group with three deuteriums. This deuterium population would be derived directly from the acetate methyl group with no isotope exchange. The second population (45% deuterated) consisted of one labeled hydrogen for each even carbon. This second population was derived from acetate via malonyl-CoA. The third population (19% deuterated) consisted of one labeled hydrogen for each odd carbon and was derived indirectly from acetate via NADPH. Desaturation of the deuterated C_{16} saturated fatty acid using the *Corynebacterium diphtheriae* desaturation system produced palmitoleic acid which lost hydrogen equivalent to one proton containing ~22% deuterium. Chemical and mass spectrometric analyses of derivatives of this palmitoleic acid clearly showed that the NADPH-derived hydrogen on C_9 was lost in the desaturation and that the acetate-derived hydrogen on C_{10} was retained. Thus, the hydrogen incorporated from acetate at C_{10} has an *L* configuration and that derived from NADPH at C_9 has a *D* configuration.

Information on the origins and stereochemistries of the hydrogen atoms that are incorporated into fatty acids during their biosynthesis has been limited. Experimental evidence has shown that hydrogens from the following sources are incorporated into fatty acids to varying degrees: methyl hydrogen atoms of acetyl-CoA (Bressler & Wakil, 1961; D'Adamo et al., 1961; Foster & Bloom, 1962), methylene hydrogens of malonyl-CoA (Bressler & Wakil, 1961; Arnstadt et al., 1975), methylene hydrogens of malonyl-ACP (Arnstadt et al., 1975), hydride ions from NADPH (Foster & Bloom, 1961; Seyama et al., 1977a,b, 1978), and the protons from water (Schoenheimer & Rittenberg, 1936; Jungas, 1968; Patton & Lowenstein, 1979). In only a few of these studies has the stoichiometry of the incorporated label at specific carbons been determined. These include the work of Jungas (1968), who determined, using adipose tissue incubated in tritiated water, that 0.96 atom and 0.71 atom of tritium/carbon atoms are incorporated on the even and odd carbons of the biosynthesized fatty acids, respectively. Particularly noteworthy is the work of Seyama et al. (1978), who have shown, using purified fatty acid synthetases, the position of deuterium incorporation into fatty acids from both deuterated water and stereospecifically deuterated pyridine nucleotides. Although these works establish the positions of the hydrogens that are incorporated into fatty acids from water and from pyridine nucleotides, they say little about the efficiencies of these incorporations and nothing about the stereochemistries of the incorporated hydrogens.

In this paper I describe the chemical, mathematical, and biosynthetic approaches which have been used to establish both the stereochemistry and stoichiometry of hydrogens incorporated in vivo into fatty acids. In the paper which follows

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(White, 1980), this information will be used to establish the stereochemistry of the sulfur insertion reaction occurring during lipoic acid biosynthesis.

Experimental Section

Materials

The Me_3Si reagent used in this work was a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (9:3:1). The casamino acids used were obtained from Difco Laboratories and contained 10% nitrogen. $^2H_2SO_4$ was obtained by the addition of SO_3 to ^2H_2O. Sodium [2-^{13}C]acetate (90 atom % ^{13}C) was obtained from Merck Sharp & Dohme of Canada.

Methods

Maintenance and Growth of Escherichia coli B. *E. coli* B was grown in 200 mL of a defined liquid medium as previously described (White, 1978) except that 6 g/L acetate was added instead of 4 g/L. Stock cultures which were maintained on agar slants of the defined medium were used to inoculate the growth medium. The cells were then grown at 37 °C for 48 h.

Isolation of Saturated Fatty Acids from E. coli B. Fresh cells (~8 g) were isolated from three growth flasks (200 mL each) by centrifugation (5000*g*; 10 min). The resulting cell pellet was resuspended in 12 mL of 8 M HCl and heated at 120 °C for 2 h. (This procedure not only frees the fatty acids from the complex lipids but also appears to decompose the cyclopropane fatty acids which interfere with the final GC–MS analysis.) After cooling, the black residue was shaken with 30 mL of methylene chloride and centrifuged at 15000*g* for 30 min to break the black emulsion. The resulting clear methylene chloride layer was removed, concentrated to ~1 mL, and mixed with 0.5 mL of saturated sodium bicarbonate and 0.5 mL of water. After centrifugation at 15000*g* for 10 min, the fatty acid salts appeared as a white insoluble layer between the water and methylene chloride phases. After removal of the clear water and methylene chloride phases, the insoluble layer was treated with 1 mL of 6 M HCl, and the free fatty acids were extracted with methylene chloride.

The resulting fatty acids (67 mg) were converted into methyl esters by reaction with diazomethane in ether. After evaporation of the solvent, the saturated esters were separated from the unsaturated esters by treatment with 200 mg of mercuric acetate in 5 mL of methanol for 2 h at 65 °C (Kishimoto & Radin, 1959). The solvent was evaporated, the residue was applied to a silicic acid column (6 × 70 mm), and the saturated fatty acid methyl esters were eluted with 6% diethyl ether in hexane (Fulco & Bloch, 1964). Pure palmitic acid was separated from the mixture of fatty acids by preparative gas chromatography of the methyl ester.

Desaturation of Saturated Fatty Acids by Corynebacterium diphtheriae. Cultures of *C. diphtheriae* were maintained on Bacto stock culture agar at 30 °C. Desaturation was done by cells growing on a liquid medium containing 27 g of casamino acids, 20 g of maltose, 500 mg of L-glutamic acid, 100 mg of L-tryptophan, 200 mg of cysteine, 140 mg of calcium chloride, and 2 mL of solution II [Mueller & Miller (1941); supplemented with 20 mg of $FeSO_4$ per 100 mL] per L. The medium was sterilized by filtration.

The deuterated fatty acids were added to 200 mL of this medium in a minimal volume (<0.5 mL) of 90% ethanol. After inoculation of the medium with bacteria from a 1-day-old slant, the cultures are grown in the presence of the deuterated fatty acids for 25 h at 30 °C on a rotary shaker. The resulting bacteria were isolated by centrifugation and digested with 20% KOH in 50% methanol for 3 h. After cooling and acidification, the fatty acids were extracted with diethyl ether and converted into methyl esters with diazomethane. The saturated and monounsaturated fatty acid methyl esters were separated on silver nitrate impregnated silicic acid TLC plates by using 6% diethyl ether in hexane as the developing solvent.

Diimide Reduction of Monosaturated Fatty Acid Methyl Esters. To each milligram of the unsaturated fatty acid methyl ester to be reduced was added 0.3 mL of a solution of 920 mg of hydrazine hydrate in 3 mL of methanol, 3 mL of pyridine, and ~10 μg of CuOAc (Schroepfer & Bloch, 1965). The solution was stirred by using a small stream of oxygen gas at room temperature for 3 h. Under these reduction conditions a known sample of methyl oleate was reduced about 50% to methyl stearate. After acidification, the reaction mixture was extracted 3 times with diethyl ether, and the resulting fatty acids were converted into methyl esters with diazomethane. The saturated and unsaturated esters were again separated by chromatography on silver nitrate impregnated TLC plates.

Preparation of the Bis[(trimethylsilyl)oxy] Derivatives of Unsaturated Fatty Acid Methyl Esters. The monounsaturated fatty acid methyl esters were converted into the diol esters as described by Eglinton & Hunneman (1968). The fatty acid methyl ester diols were converted into the trimethylsilyl derivatives by reaction with the Me_3Si reagent at 60 °C for 15 min prior to GC–MS analysis.

Cleavage of Unsaturated Fatty Acid Methyl Esters. The isolated monounsaturated fatty acid methyl esters were cleaved in 30% *tert*-butyl alcohol by using the periodate–permanganate system described by Von Rudloff (1956). At the end of the reaction (2 h), solid $NaHSO_3$ was added to reduce iodine to iodide, and, after acidification, the cleavage products were extracted with methylene chloride. The dried methylene chloride extract was reacted with the Me_3Si reagent prior to GC–MS analysis.

Preparation of [2,2′,10L-$^2H_3$]Palmitic Acid. Methyl 12*D*-hydroxystearate was prepared by catalytic reduction of methyl ricinoleate with 5% Pd/C. The tosylate of the hydroxy fatty acid was prepared as described by Fieser & Fieser (1967), and it was reduced to [2,2′,12*L*-$^2H_3$]stearyl alcohol by heating with excess $LiAl^2H_4$ in tetrahydrofuran at 65 °C for 25 h (Schroepfer & Bloch, 1965). After workup of the reaction, the labeled stearyl alcohol was isolated by column chromatography on silica gel and oxidized to the acid with chromic acid as described by Pattison et al. (1956). The resulting stearic acid was then shortened by two carbon atoms by α-bromination, followed by elimination to the α,β-unsaturated acid as described by Allen & Kalm (1963) and subsequent cleavage of the unsaturated acid with periodate–permanganate as described by Von Rudloff (1956). The resulting product was labeled at C_2 by heating with 90% $^2H_2SO_4$ as described by Van Heyningen et al. (1938). The final product, after conversion to the methyl ester, had small amounts of C_{14} and C_{18} fatty acids which were removed by preparative gas chromatography.

Gas Chromatography–Mass Spectrometry. An LKB 9000 S gas chromatograph–mass spectrometer equipped with either a 4 ft × 1/8 in. column packed with 3% OV-1 on Gas-Chrom Q or a 6 ft × 1/8 in. column packed with 3% OV-17 on Gas-Chrom Q was used for the analyses described herein. The operating conditions and methods used for the evaluation of the isotopic abundances have been previously described (White, 1978). The fatty acid methyl esters were separated in the

OV-1 column by temperature programming from 100 °C at 8 °C/min.

Calculation and Data Evaluation. The calculated distribution of the deuterium for a given fatty acid was obtained by expanding the polynomial described under Results. The experimentally observed isotopic distribution was obtained by subtracting the experimentally observed natural isotopic abundance, determined from unlabeled samples from the observed isotopic abundances of the biosynthetic samples as outlined by Biemann (1962).

The best fit between the calculated and observed data was determined by a statistically weighted least-squares analysis. The difference in the percent abundance at each mass increment for each set of data was determined. This was then multiplied by the reciprocal of the observed data for that mass increment and the product was squared. The best fit between the two sets of data was established when the sum of these values reached a minimum, as the coefficients used to generate the calculated data were varied.

Results

The distribution of deuterium found in a fatty acid biosynthesized from [*methyl*-$^2H_3$]acetate would be expected, to a first approximation, to fit the coefficients generated by expanding the expression

$$(a + b)(c + d)^{n-1}(x + y)^{n-1}$$

where the ratio $b/(a + b)$ represents the percent of molecules with three deuterium in the methyl group, the ratio $d/(c + d)$ represents the percent of deuteration for the single hydrogen on the odd carbons derived from acetate, the ratio $y/(x + y)$ represents the percent of deuteration for the single hydrogen on the even carbons derived from deuterated NADPH, and n represents the number of acetate units in the fatty acid. A full description of the use of this formula for the evaluation of isotopic peaks has been given by Biemann (1962).

The b, d, and y values, which give the observed deuterium isotope patterns for a given fatty acid, can be evaluated by several independent methods of analysis. The most direct of these methods is simply to determine which values, when expanded in this expression, give the best fit to the observed experimental data for the fatty acids. The best fit was established with $b = 0.8$, $d = 0.48$, and $y = 0.19$. A comparison between the calculated deuterium distribution using these values and the observed deuterium distribution for each fatty acid is shown in Table I. The observed deuterium distributions were obtained from the normalized ion intensities also shown in Table I after correction for the natural isotopic abundances.

The values for d and y can also be determined by establishing what deuterium populations at two different hydrogen sites must be added to the data of one fatty acid to generate the data for the next higher homologue. By performance of this calculation, it can be shown that for the best conversion of the lauric acid data to the myristic acid data one hydrogen containing 46% deuterium and one hydrogen containing 19% deuterium must be added. Likewise, for the best conversion of the myristic acid data to the palmitic acid data, one hydrogen containing 45% deuterium and one hydrogen containing 21% deuterium must be added. These calculated distributions are also shown in Table I. These results agree with the d and y values determined by the first method.

The d value can be independently derived by measuring the amount of deuteriums present in the m/e 74 ion for each of the saturated fatty acids. This ion results from a McLafferty rearrangement of the intact fatty acid methyl ester and contains all of the C_2 hydrogens and one of the C_4 hydrogens

Table I: Comparison of the Observed Deuterium Distribution in the Biosynthesized Fatty Acids with the Calculated Values

fatty acid	no. of ^2H:	deuterium distribution (%)															
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
lauric acid	obsd[a]	3.9	6.6	13.5	29.0	55.2	85.8	100	83.5	52.5	24.5	8.9	2.4	0.66				
	obsd[b]	0.30	1.5	3.2	6.8	12.9	19.6	22.3	17.4	10.2	4.3	1.4	0.31	0.065				
	calcd[c]	0.30	1.5	3.8		10.9	18.1	22.8	19.5	11.1	4.2	1.1	0.16	0.001				
myristic acid	obsd[a]	3.9	4.7	10.1	23.0	47.5	75.9	99.7	100	80.0	49.7	24.3	9.5	2.9	0.9	0.2		
	obsd[b]	0.15	0.92	2.1	4.8	9.9	15.3	19.7	18.9	14.4	8.4	3.8	1.3	0.36	~0.11	~0.007		
	calcd[c]	0.11	0.77	2.3	4.7	8.1	13.5	19.4	21.0	16.3	8.9	3.5	1.0	0.18	0.020	0.001		
	calcd[d]	0.14	0.82	2.1	4.7	9.3	15.3	20.2	19.8	14.6	8.2	3.6	1.1	0.31	0.070	0.008		
palmitic acid	obsd[a]	4.7	4.8	9.9	19.8	37.2	63.1	88.1	100	94.4	71.8	45.4	23.9	10.0	3.3	1.1	0.3	0.05
	obsd[b]	0.12	0.84	1.9	3.9	7.1	11.9	16.2	17.8	16.2	11.8	7.1	3.5	1.3	0.38	0.14	~0.031	~0.002
	calcd[c]	0.05	0.38	1.4	3.2	5.9	10.1	15.6	19.6	18.9	13.6	7.3	2.9	0.9	0.18	0.03	0.0003	0.000002
	calcd[e]	0.07	0.47	1.4	3.2	6.7	11.7	16.6	18.9	17.1	12.3	7.2	3.3	1.2	0.35	0.09	0.014	0.0002

[a] Observed normalized ion intensities measured from the molecular ion of the methyl ester of each fatty acid. [b] Data have been corrected for the naturally occurring isotope abundances and are reported as the percent of the total molecules containing the indicated number of deuteriums. [c] Calculated by using $b = 0.8$, $d = 0.48$, $y = 0.19$, and the appropriate expansion for each fatty acid. [d] Calculated from the lauric acid data by adding one hydrogen containing 46% deuterium and one hydrogen containing 0.19% deuterium. [e] Calculated from the myristic acid data by adding one hydrogen containing 45% deuterium and one hydrogen containing 21% deuterium.

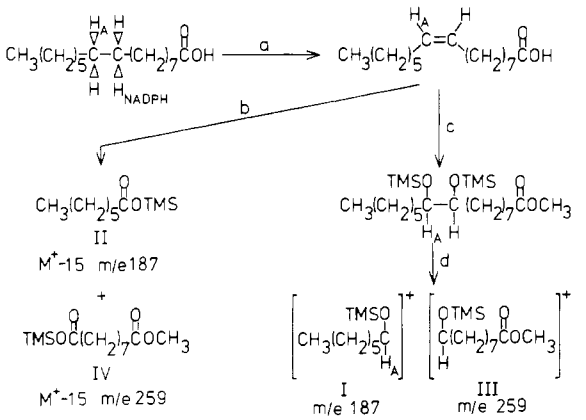

FIGURE 1: Outline of the chemical and biological methods and mass spectral fragments used to establish the stoichiometry and stereochemistry of deuterium incorporated at C_9 and C_{10} of palmitic acid. (a) Stereospecific desaturation of the C_9 and C_{10} hydrogens using cultures of *C. diphtheriae*; (b) cleavage of the desaturated palmitoleic acid with $KMnO_4/KIO_4$; (c) oxidation of the desaturated palmitoleic acid with OsO_4; (d) a 70-eV mass spectrum was used to generate fragments I and II.

(Dinh-Nguyen et al., 1961). As a result of the incorporation of the C_4 hydrogen into this ion, the deuterium incorporated both at C_2 and C_4 can be estimated. Measurement of the deuterium incorporated into the *m/e* 74 ion for each of the fatty acids showed the ion to contain two separate populations of deuterium, one containing 40–45% deuterium and a second containing half of this amount. Since only one of the two hydrogens is removed from C_4, the data is consistent with a 40–45% incorporation of deuterium on both the C_2 and C_4 of each of the fatty acids.

By a similar analysis, a *y* value of 20% was determined for the hydrogens on C_{11} and C_{13} in palmitic acid. In this case, the *m/e* 74 ion in the mass spectrum of methyl heptanoate, chemically derived from palmitic acid as shown in Figure 1, was used.

The value for *y* was also determined by establishing the extent of deuteration of the NADPH in the cells over the time course of the growth. This was done by determining the incorporation of deuterium into amino acids which use NADPH in their biosynthesis. The amino acids proline and threonine were selected for this work because each uses two NADPH in their biosynthesis. Thus, by comparing the deuterium distribution in the glutamic acid with that of its biosynthetic product proline, the percent of deuterium in the cellular NADPH can be calculated. In a similar manner the difference between the deuterium distribution in the threonine and the distribution in its biosynthetic precursor aspartic acid also allows for a calculation of the extent of deuteration of the cellular NADPH. Both of the measurements show that the cells' NADPH contained 19% deuterium (White, 1980).

Finally, the *b* value can be determined by establishing the extent of incorporation of the acetate C_2 carbon into the fatty acids. When the cells were grown with acetate labeled at C_2 with 18% ^{13}C, 15% ^{13}C was incorporated for each acetate. Thus, the fed acetate methyl was incorporated at about 83%. Since the terminal methyl group should be incorporated with little loss of deuterium, this data supports the calculated *b* value of 80%.

After establishment of the overall pattern of deuterium incorporation, the next step was to determine the stereochemistry of the incorporated deuterium. This was accomplished by the desaturation of the palmitic acid using the cultures of *C. diphtheriae* as described by Schroepfer & Bloch (1965). In this work, it was established that the 9*D* and 10*D* hydrogens of stearic acid are removed during its desaturation to oleic acid. Thus, by measuring the loss or retention of the deuterium on C_9 and C_{10} of the biosynthetically derived stearic acid, the stereochemistry of the deuterium atoms incorporated at these two carbons can be established. However, since the *E. coli* strain used in this study only had very small amounts of stearic acid, it first had to be established that the same stereospecific desaturation would occur at the same carbons of palmitic acid, which is the major saturated fatty acid in these cells. From Table III, it can be seen that *C. diphtheriae* readily desaturated the [2,2′,10*L*-$^2H_3$]palmitic acid to palmitoleic acid with retention of the three deuteriums. After correction for the isotope peaks from the nonlabeled acid, it was found that the ratio of palmitoleic acids having two and three deuteriums remained constant during the desaturation thereby establishing that the 10*L*-deuterium was retained in the desaturation. Since the desaturation takes place at carbons 9 and 10, as will be shown later, then the 10*D* hydrogen is removed during desaturation. Although no work was done here with stereospecifically labeled C_9 palmitic acids, it is clear from the work of Schroepfer & Bloch (1965) that *D* hydrogen must also be removed from this carbon.

The results from the first desaturation of the biosynthetically deuterated palmitic acid are reported in Table II. As expected, this data is complicated because all the carbons are deuterated, not just the C_9 and C_{10}. However, it is clear from the data that there is a decrease in the amount of deuterium in the isolated palmitoleic acid as compared to the fed palmitic acid. By considering what the deuterium enrichment at one site would have to be to give this amount of shift in the isotopic distribution, one can calculate that one hydrogen containing 22% deuterium would have to be lost in the desaturation. (This calculation is based on the ion intensities for ions containing 7–12 deuterium atoms because the ion intensities are more intense in this range and they are not contaminated by the nonlabeled palmitoleic acid present in the sample.) It is also apparent that a substantial isotope fractionation has occurred during the desaturation in that the deuterated palmitic acid isolated from the cells shows an enrichment in the amount of deuterium.

This enrichment appears to be a result of two separate types of isotope effects. The first is a specific isotope effect against the removal of the *D* deuterium on C_9. This isotope effect was first observed by Schroepfer & Bloch (1965) in their work on the desaturation of stearic acid. Since this deuterium is always removed for each molecule desaturated, its only effect would be to increase the average amount of deuterium in the nondesaturated palmitic acid.

The second isotope effect will be referred to as a mass isotope effect. This effect results from the more likely desaturation of the less deuterated molecules, and, as in the specific isotope effect, it not only increases the deuterium in the nondesaturated palmitic acid but also produces a decrease in the amount of deuterium in the desaturated fatty acid over that directly lost in the desaturation. This second isotope effect would be expected to become more pronounced as increased amounts of the deuterated fatty acids are fed because there would be a corresponding increase in the amount of the less deuterated molecules. The true cause for this effect is not completely clear, but it may be due to a decrease in the diffusion rate of the more highly deuterated fatty acids resulting in a decrease in their overall reactivity.

The feeding experiment was repeated a second time on a larger scale and using a larger amount of the deuterated fatty acids in order to increase both the amount of deuterium in-

Table II: Isotopic Distributions of Deuterated C_{16} Acids Metabolized by *C. diphtheriae*[a]

	m/e														
	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282
Synthetic 10-*L*-Deuteriopalmitic Acid															
isolated palmitic acid			100	19.9	5.9	4.6									
fed palmitic acid			1.7	13.8	66.6	100									
isolated palmitoleic acid reduced			100	21.7	7.8	7.8									
isolated palmitoleic acid	100	21.7	7.8	7.8											
Biosynthetic Deuterated Palmitic Acids															
isolated palmitic acid			2850	729	87.9	24.3	30.8	54.2	78.5	96.1	100	76.2	49.2	28.2	11.7
fed palmitic acid			4.7	4.8	9.9	19.8	37.2	63.1	88.1	100	94.4	71.8	45.1	23.9	10.0
isolated palmitoleic acid reduced			2305	587	81.5	27.2	38.6	61.2	86.8	100	95.1	65	41	19.8	7.3
isolated palmitoleic acid	2535	549	71	26.7	40.0	65.5	82.7	100	98.9	66.1	41.1	21.8	7.3		

[a] One-milligram samples of the pure C_{16} fatty acids were added to *C. diphtheriae* growing in 50 mL of culture medium. At the end of log-phase growth the fatty acids were extracted from the bacteria and their isotopic distribution was determined by GC–MS of the methyl esters. Ion intensities were measured from the molecular ions and were normalized with the deuterium-containing ion of highest intensity being 100%.

Table III: Isotopic Distributions of Deuterated C_{16} Fatty Acids Metabolized by *C. diphtheriae*[a]

	m/e															
fatty acid	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283
isolated palmitic acid			93.8	22.3	10.6	17.6	34.1	58.5	86.0	100	95.5	72.7	46.6	24.5	9.9	3.7
fed palmitic acid			4.7	4.8	9.9	19.8	37.2	63.1	88.1	100	94.4	71.8	45.4	23.9	10.0	3.3
isolated palmitoleic acid reduced			689	134	24.2	21.7	39.8	64.8	92.3	100	90.3	62.7	36.1	17.6	7.1	1.8
isolated palmitoleic acid	681	148	27.0	20.3	38.8	64.0	90.8	100	89.6	62.1	36.1	17.5	6.3	1.8		

[a] *E. coli* produced saturated deuterated fatty acids (23.6 mg) containing 54% palmitic acid were added to 200 mL of growth medium. Cells were extracted, and the C_{16} fatty acids were assayed as described in Table II.

corporated into the desaturated fatty acid as well as to supply more material. The results of this feeding are shown in Table III. These data indicate that one hydrogen containing 25% deuterium must be added to the desaturated palmitoleic acid data to generate the same isotopic peak distribution as found in the fed palmitic acid data. The slight increase in the percent of deuterium removed (3%) is a result of the mass isotope effect discussed earlier. The much smaller increase in the amount of deuterium in the isolated palmitic acid, as compared to the first feeding, results simply from the much larger amount of labeled palmitic acid present in the cell which masks the isotopic enrichment.

Given only that the equivalent of a single hydrogen containing ~22% deuterium is lost in the desaturation, it is impossible to make any definitive statement about the stereochemistry of the deuterium at either C_9 or C_{10} as a wide choice of possibilities may exist which are consistent with this data. For example, there might be a random incorporation of deuterium at C_{10} with the NADPH deuterium incorporated in the *L* configuration at C_9. Desaturation of this system would show that a single hydrogen containing ~22% deuterium was lost. This same loss would be observed if the C_9 had 22% deuterium in the *D* position and C_{10} had none.

Thus, it had to be established unequivocally from which carbons of the deuterated palmitic acid this loss of deuterium occurred. This was done by comparing the extent of labeling in fragments of the palmitoleic acid which either retained or lost all of the hydrogens at C_9 and C_{10}. Figure 1 outlines the chemical methods and mass spectral fragments used for this comparison. It is clear that the difference between the deuterium content of compound II and that of the Me_3Si fragment I is the amount of deuterium remaining in C_{10} after the desaturation. Similarly, the difference between the deuterium content of compound IV and that of the Me_3Si fragment III represents the deuterium present on C_9 after the desaturation.

Table IV shows the results obtained from fragments I and II. The data clearly indicate that a substantial loss in deuterium has resulted from the removal of all hydrogens from C_{10}. By calculation of the deuterium enrichment at C_{10} required to change the isotopic distribution of II to I, a value of 45% is obtained. Since the C_{10} of the palmitoleic acid has only one hydrogen and the *D* hydrogen was removed in the desaturation, then all of the original acetate deuterium must have been incorporated in the *L* configuration. Since this leaves no room for any deuterium loss at C_{10} in the original desaturation, then all of the deuterium loss must have occurred from the deuterium incorporated in the *D* configuration at C_9 from NADPH. If this is correct, then we would expect to find no deuterium on the C_9 of the desaturated palmitoleic acid. This can be substantiated by comparing the deuterium distribution in fragment III with that of fragment IV. If no deuterium is present at C_9, then we would expect both of these fragments to have the same deuterium distribution. This is confirmed by the finding of no difference in the deuterium distributions of fragments III and IV which are reported in Table V.

Discussion

In addition to supplying the desired stereochemical information for subsequent studies on lipoic acid biosynthesis, the

Table IV: Observed Distribution of Deuterium in the C_{10}- to C_{16}-Containing Fragments of the Desaturated Palmitic Acid[a]

fragment	row	no. of ^2H:	deuterium distribution (%) 0	1	2	3	4	5	6	7	8	9
$[CH_3(CH_2)_5C(H)(OTMS)]^+$	1[c]		1.7[b]	5.4	7.3	14.5	24.5	26.4	14.5	5.0	0.70	0.10
	2[d]		1.7[b]	5.5	7.0	14.5	24.9	25.3	14.9	5.0	0.98	0.09
	3[f]		1.7	5.5	6.9	11.1	23.2	27.7	17.3	5.6	0.91	0.06
	4[g]		1.8	5.6	7.8	13.2	24.2	26.4	15.3	4.7	0.72	0.04
I, *m/e* 187	5[h]		1.7	5.4	6.3	13.5	24.8	25.9	15.3	5.1	0.85	0.06
	6[c]		3.1[b]	7.3	7.2	18.7	29.8	22.7	9.3	1.7	0.14	
$CH_3(CH_2)_5C(=O)OTMS$	7[e]		3.0[b]	7.2	7.6	18.8	29.3	23.3	8.7	1.8	0.20	
II, *m/e* 187 ($M^+ - 15$)	8[i]		3.1	7.4	6.5	14.9	30.0	25.9	10.2	1.9	0.13	

[a] Data have been corrected for the natural isotopic abundances and the contribution from the unlabeled palmitoleic acid present in the sample. Data are expressed as the percentage of the total fragment containing the indicated number of deuteriums. [b] Estimated value based on the percentage of the molecules having five deuteriums and on the calculated data from rows 3[f] and 8[i]. [c] Observed data determined by using an OV-1 column for gas chromatography. [d] Observed data determined by using an OV-17 column for gas chromatography and an independently derivatized sample from that used in row 1[c]. [e] Observed data determined by using an OV-17 column for gas chromatography. [f] Calculated with $b = 0.8$, $d = 0.45$, $y = 0.2$, one methyl (CD_3) "b" group, three "d" hydrogens, and three "y" hydrogens. [g] Calculated the same as row 3[f] but by assuming the methyl group to have 10% with two deuteriums and 70% with three deuteriums. [h] Calculated from the data in row 6[c] by adding one hydrogen containing 45% deuterium. [i] Calculated with $b = 0.8$, $d = 0.45$, $y = 0.20$, and one methyl (CD_3) "b" group, two "d" hydrogens, and three "y" hydrogens.

Table V: Observed Distribution of Deuterium in the C_1- to C_9-Containing Fragments of the Desaturated Palmitic Acid[a]

fragment	row	no. of ^2H:	deuterium distribution (%) 0	1	2	3	4	5	6	7
$[CH_3OC(=O)(CH_2)_7C(H)(OTMS)]^+$	1[c]		4.5[b]	20.2	30.1	26.1	13.3	4.1	1.5	0.21
	2[d]		4.3[b]	21.9	29.2	25.9	12.5	4.8	1.2	0.12
	3[e]		4.3	17.4	29.4	27.4	15.2	5.2	1.1	0.12
III, *m/e* 259										
$CH_3OC(=O)(CH_2)_7C(=O)OTMS$	4[c]		5.1[b]	20.3	29.4	25.9	13.6	4.6	0.86	0.22
IV, *m/e* 259 ($M^+ - 15$)										

[a] Data have been corrected for the natural isotopic abundances and the contribution from the unlabeled palmitoleic acid present in the sample. Data are expressed as the percentage of the total fragment containing the indicated number of deuteriums. [b] Estimated value based on the percentage of the molecules having two deuteriums calculated from the data in row 3[e]. [c] Observed data determined by using an OV-1 column for gas chromatography. [d] Observed data determined by using an OV-17 column for gas chromatography and an independently derivatized sample from that used in row 1[c]. [e] Calculated values with $d = 0.43$, $y = 0.2$, four "d" hydrogens, and four "y" hydrogens.

data presented here have some interesting implications concerning fatty acid biosynthesis in *E. coli*. The most surprising finding is that no deuterium appears to be incorporated in vivo during the enoyl reductase step of fatty acid biosynthesis. The β-ketoacyl reductase step which is specific for NADPH (Toomey & Wakil, 1966) fully accounts for all the deuterium incorporated from both of the reductive steps. The enoyl-[acyl-carrier-protein] reductase has been isolated by Weeks & Wakil (1968), who presented evidence that the activity is due to two separate enzymes, one specific for NADPH and one specific for NADH.

Since both pyridine nucleotides are labeled with deuterium (White, 1980), then some mechanism must be operating which prevents the incorporation of deuterium at the enoyl reductase step. This could be explained by the intermediacy of a flavin in the enoyl reductase step or by the rapid exchange of the pyridine nucleotide hydrogens catalyzed by the enoyl reductase as observed by Seyama et al. (1977a,b). It is clear, however, that the stereochemical course of this reduction will be the only factor in determining the stereochemistry of the incorporated deuterium derived from both acetate and NADPH. The observed stereochemistry can best be explained by an apparent "cis" addition of hydrogen to the *L* side of the trans double bond of the enoyl-ACP. (It should, however, be pointed out that the experimental data only prove the final configuration of the incorporated deuteriums and that they do not prove that the hydrogen addition at the enoyl reductase step occurs by a cis addition.) Precedent for this "cis" addition, as well as for the lack of hydrogen incorporation, comes from the work of Hunter et al. (1976) on the biohydrogenation of the *cis*-9,*trans*-11,*cis*-13-octadecatrienoic acid and from the work of Morris (1970) on the biohydrogenation of oleic and elaidic acids.

Finally, the fact that only about half of the expected amount of deuterium is incorporated from each acetate should be considered. Since it has recently been shown (Arnstadt et al., 1975) that the β-ketoacyl-[acyl-carrier-protein] synthetase from *E. coli* has no kinetic isotope effect with dideuteriomalonyl-ACP, then this reduction in the deuterium incorporation must result from either a direct exchange from malonyl-SCoA and malonyl-ACP and/or a kinetic isotope effect on the acetyl-CoA carboxylase reaction. The present data, however, do not allow the contributions from each of these effects to be established.

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Stable Isotope Studies on the Biosynthesis of Lipoic Acid in *Escherichia coli*†

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ABSTRACT: A method has been developed for the gas chromatographic–mass spectrometric (GC–MS) identification of lipoic acid in tissue. The method consists of acid hydrolysis of the tissue to free the bound lipoic acid, methylene chloride extraction of the lipoic acid, and subsequent chemical derivatization of the lipoic acid as methyl 6,8-bis(benzylthio)octanoate prior to GC–MS analysis. By use of this method of analysis, the incorporation of deuterium into lipoic acid by *Escherichia coli* growing on [*methyl*-$^2H_3$]acetate has been studied. The results clearly show that the lipoic acid is biosynthesized from octanoic acid with the loss of only one deuterium-containing position at C_8. The deuterium incorporated at C_6 of octanoic acid from the labeled acetate is retained. Since this deuterium is incorporated in the L configuration during fatty acid biosynthesis and it is known to have the D configuration in lipoic acid, it is concluded that an inversion of configuration occurs at C_6 during the sulfur insertion.

Despite the fact that the structure of lipoic acid has been known for over 30 years, no method for its microchemical analysis has ever been devised. This is, perhaps, partly responsible for the lack of work done on its biosynthesis. However, other factors such as the exceedingly small amounts of lipoic acid present in tissues and the fact that no mutant has ever been isolated which overproduces the substance must also have contributed.

A method is described in this paper for the GC–MS analysis of lipoic acid—a method which surmounts these problems. Furthermore, it will be shown how this analytical method, along with stable isotopes and the natural metabolism in wild-type *Escherichia coli* cells, can be used to study the biosynthesis of lipoic acid. The methodology described herein represents an extension of the metabolic labeling technique previously outlined by the author (White, 1978).

Experimental Section

Methods

Maintenance and Growth of the Organism. E. coli B was grown on [*methyl*-$^2H_3$]acetate and casamino acids as described in the preceding paper (White, 1980). Cultures were grown

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