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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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in media containing either 4, 6, or 8 g/L of the labeled acetate. In the 4 and 8 g/L acetate media, a synthetic mixture of L-amino acids with the same composition as the casamino acids was used but with the omission of serine, threonine, and proline.

The [U-$^2H_{15}$]octanoic acid feeding was conducted in 400 mL of the culture medium containing 2 g of glucose and 2 g of casamino acids. Forty milligrams of the labeled octanoic acid was added to the medium at inoculation. Growth was continued for 16 h at 37 °C.

*Method for the Isolation and GC–MS Analysis of Lipoic Acid.* The cells were isolated, hydrolyzed, and extracted as described in the preceding paper for the isolation of fatty acids (White, 1980). The resulting clear sodium bicarbonate extract (1 mL) from the above procedure was cooled to 3 °C. Thirty milligrams of sodium borohydride was dissolved in this solution, and this was followed by the addition of 1 mL of methanol and 10 μL of benzyl chloride. The reaction mixture was then placed in a sealed vial and heated at 60 °C for 30 min with shaking and periodic venting of the hydrogen gas. At the end of this period, 1 mL of 6 M HCl was added to the cooled solution which was then extracted 3 times with 2-mL portions of methylene chloride. The combined methylene chloride extracts were then treated for 10 min with an excess of diazomethane and evaporated to dryness. The residue was dissolved in 50 μL of ethyl acetate for GC–MS analysis.

*Preparation of Methyl [6,8,8′-$^2H_3$]-6,8-Bis([α,α′-$^2H_2$]-benzylthio)octanoate.* 6,8-Bis(benzylthio)octanoic acid was prepared by the reaction of reduced lipoic acid with benzyl chloride as described in the preceding paragraph. The resulting product was converted into 6,8-bis(benzylsulfoxyl)octanoic acid by periodate oxidation in 50% methanol as described by Leonard & Johnson (1962). The 6,8-bis(benzylsulfoxyl)octanoic acid was exchange-labeled by heating at 100 °C for 12 h in $D_2O$ containing 1 equiv of sodium carbonate. The resulting [6,8,8′-$^2H_3$]-6,8-bis([α,α′-$^2H_2$]benzylsulfoxyl)octanoic acid was converted to [6,8,8′-$^2H_3$]-6,8-bis([α,α,′-$^2H_2$]benzylthio)octanoic acid by reduction with triphenylphosphine in carbon tetrachloride as described by Castrillon & Szmant (1965). GC–MS of this final product as the methyl ester demonstrated that seven deuteriums had been incorporated. This is the expected result as all hydrogens on carbons α to the sulfoxide groups are exchangeable.

*Analysis of the Deuterium in the 4 Position of NADH.* The average percentage of deuterium in the 4 position of the nicotinamide ring of NADH, present in the cell over its entire growth cycle, can be obtained by measuring the deuterium incorporated into the biosynthesized glycerol-P (White, 1978). Glycerol-P biosynthesis in *E. coli* grown on acetate begins with 1,3-diphospho-D-glycerate which is converted into glycerol-P by three separate reactions, two of which incorporate a hydrogen from NADH. In the first of these reactions, catalyzed by glyceraldehyde-P dehydrogenase, 1,3-diphospho-D-glycerate is converted to D-glyceraldehyde 3-P with the incorporation of one NADH hydrogen at $C_3$ of the glyceraldehyde 3-P. (This deuterium will appear at the 3 position of glycerol-P.) After conversion of the glyceraldehyde 3-P to dihydroxyacetone-P, a second NADH-dependent reduction catalyzed by glycerol-P dehydrogenase converts the dihydroxyacetone-P to glycerol-P.

This last reduction incorporates a second deuterium from NADH into the $C_2$ position of the glycerol-P. The only other position where a deuterium could be incorporated into glycerol-P would be at $C_1$. This would result from the deuterium being incorporated into the $C_1$ position of 1,3-diphospho-D-glycerate from acetate. The extent of labeling of this position can be evaluated by analyzing the deuterium incorporated into serine, in which the $C_3$ hydrogens have the same origin as the $C_1$ hydrogens of the glycerol-P. By subtraction of the amount of label found at $C_1$ from that in the entire molecule, the sum of the amount of deuterium at $C_2$ and $C_3$ can be obtained. Furthermore, by evaluation of the deuterium distributions in the fragments of the $Me_3Si$ derivative of the glycerol-P (White, 1978), the exact amounts of deuterium incorporated at $C_2$ and $C_3$ can be independently determined. These analyses show that in the growth media with 4 and 8 g/L acetate, 11 and 12% deuterium are incorporated at both $C_2$ and $C_3$, respectively.

*Analysis of the Deuterium in the 4 Position of NADPH.* The percentage of deuterium in the NADPH in the cell averaged over the entire growth cycle was established by measuring the deuterium incorporated into the threonine and proline biosynthesized by the cells. These two amino acids are synthesized from aspartic acid and glutamic acid, respectively, by two NADPH-dependent reductions (Truffa-Bachi & Cohen, 1970; Csonka & Fraenkel, 1977). By establishment of the difference between the deuterium content of cellular threonine and aspartic acid and between that of the cellular proline and glutamic acid, the extent of deuteration of the NADPH can be established. The procedures used to determine the deuterium levels in the proline and threonine in the cell were performed as previously described (White, 1978).

Extensive hydrogen exchange at the methylene groups adjacent to the side chains of the aspartic and glutamic acids during 6 M HCl hydrolysis prevented the direct measurement of the deuterium incorporated into these amino acids. The extent of deuteration in these amino acids was obtained by measuring the loss of deuterium from each amino acid, as it was released from the cellular protein during acid hydrolysis. The isotopic distribution for each amino acid was then determined by extrapolating the measured deuterium incorporation at several time points during the hydrolysis back to zero time. The isotopic distribution of the released amino acids was assayed by GC–MS as the $Me_3Si$ derivative.

*Gas Chromatography–Mass Spectrometry.* An LKB 9000S gas chromatograph–mass spectrometer equipped with a 4 ft × 1/8 in. glass column packed with 3% OV-1 on Gas-Chrom Q (Applied Sciences, Inc.) was used for the analyses described herein. Methyl 6,8-bis(benzylthio)octanoate had a retention time of 2–3 min on this column when operated at 240 °C with a helium flow of 25 mL/min. The injector and separator were maintained at 280 and 260 °C, respectively. Octanoic acid was assayed in the fatty acid fraction where it had a retention time of 3.4 min at 100 °C.

### *Materials*

[U-$^3H_{15}$]Octanoic acid (98 atom % $^2H$) was obtained from Merck Sharp & Dohme of Canada. [*methyl*-$^3H_3$]Acetic acid and DL-lipoic acid were obtained from Sigma Chemical Co.

## Results

The isolation of lipoic acid from small amounts of tissue in a chemically identifiable form proved to be exceedingly difficult. Although pure samples of lipoic acid could be readily gas chromatographed as the methyl ester, repeated attempts to identify the lipoic acid in appropriately fractionated and derivatized samples proved futile. This lack of success may have been caused, in part, by the formation of mixed disulfides (Reed et al., 1953a) and disulfide polymers (Thomas & Reed, 1956) which is characteristic of lipoic acid. The tissue-isolated lipoic acid was converted into derivatives in which the sulfurs

Table I: Isotopic Distribution of Lipoic Acid Biosynthesized by *E. coli* Grown on [*methyl*-$^2H_3$]Acetate[a]

| [*methyl*-$^2H_3$]-acetate in growth medium | | deuterium distribution (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | no. of $^2H$: | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 4 g/L | obsd | 5.3[h] | 7.6 | 15.1 | 24.3 | 25.5 | 15.5 | 5.6 | 1.1 | 0.035 |
| | calcd[b] | 2.8 | 8.5 | 16.8 | 26.1 | 26.1 | 14.6 | 4.4 | 0.64 | 0.040 |
| | calcd[c] | 5.2 | 11.6 | 21.4 | 30.3 | 22.3 | 7.8 | 1.3 | 0.08 | 0 |
| 6 g/L | obsd | 4.8[h] | 5.7 | 15.0 | 26.2 | 26.9 | 15.5 | 5.1 | 0.93 | 0.05 |
| | calcd[d] | 2.1 | 6.8 | 14.9 | 25.9 | 27.7 | 16.5 | 5.2 | 0.82 | 0.05 |
| | calcd[e] | 3.9 | 9.3 | 19.7 | 31.1 | 24.8 | 9.4 | 1.7 | 0.11 | 0 |
| 8 g/L | obsd | 3.4[h] | 6.1 | 13.4 | 24.1 | 27.9 | 17.7 | 6.0 | 1.3 | 0.16 |
| | calcd[f] | 1.6 | 6.0 | 13.5 | 24.1 | 28.2 | 18.7 | 6.5 | 1.3 | 0.075 |
| | calcd[g] | 3.2 | 8.8 | 18.2 | 30.1 | 26.5 | 11.0 | 2.1 | 0.15 | 0 |

[a] Data have been corrected for the naturally occurring isotopic abundances and are reported as the percent of the total molecules containing the indicated number of deuteriums. [b] Calculated with $b = 0.7$, $d = 0.44$, and $y = 0.18$ by assuming the $C_6$ deuterium is retained. [c] Calculated with the same values as in footnote *b* but by assuming the $C_6$ deuterium is lost. [d] Calculated with $b = 0.75$, $c = 0.46$, and $y = 0.19$ by assuming the $C_6$ deuterium is retained. [e] Calculated with the same values as in footnote *d* but by assuming the $C_6$ deuterium is lost. [f] Calculated with $b = 0.75$, $c = 0.5$, and $y = 0.20$ by assuming the $C_6$ deuterium is retained. [g] Calculated with the same values as in footnote *f* but by assuming the $C_6$ deuterium is lost. [h] Data not corrected for the possible occurrence of a small percent (~2%) of nonbiosynthesized unlabeled lipoic acid.

were protected as sulfides in order to surmount this problem.

A series of derivatives which offered this protection were prepared by reacting reduced lipoic acid with the reagents dinitrofluorobenzene, iodoacetate, 2,2′-dimethoxypropane, and benzyl chloride under appropriate conditions. All of these derivatives, with the exception of the *S,S*-dibenzyl derivative, proved unacceptable for a GC–MS assay of lipoic acid due to either their nonvolatility, instability, or difficulty in preparation. The *S,S*-dibenzyl derivative, however, had all the characteristics desired for a GC–MS assay, it being easily prepared, quite stable, and easily chromatographed. The preparation of this product consists of the reduction of a dilute aqueous solution of lipoic acid with sodium borohydride, followed by benzylation of the reduced disulfide with benzyl chloride.

Maximization of the production of this derivative from a 1-mL solution containing 20 μg/mL lipoic acid (that calculated to be released from 10 g of cells on acid hydrolysis) was achieved by varying the time and temperature of the reaction, the percentage of methanol in the reaction mixture, the amount of sodium borohydride and benzyl chloride added, and the pH of the reaction mixture. The following conditions were found to give maximum recovery of the *S,S*-dibenzyl derivative: 30 min at 60 °C, 50% methanol in the reaction mixture, 50 mg of sodium borohydride and 10 μL of benzyl chloride added, and the pH of the reaction maintained at ~8.4 with sodium bicarbonate. The amount of the *S,S*-dibenzyl derivative formed was assayed as the methyl ester by GC after extraction of the acidified reaction mixture with methylene chloride and reaction with diazomethane. Formation of the methyl ester with diazomethane was found to be far superior to the formation of the ester with 3 M HCl in methanol at either 23 or 100 °C for 3 h.

The next phase in the development of the lipoic acid analysis by GC–MS consisted of removing the lipoic acid from the cells and separating it from the large amount of other materials present. Previous work (Reed et al., 1953b) established that the major portion of cellular lipoic acid is present in the cell in a bound form; only after hydrolysis can the lipoic acid be extracted into organic solvents. However, performing the standard procedure of 6 M acid hydrolysis, followed by extraction with methylene chloride, gave a large amount of material, the major portion of which was fatty acids. It was found, however, that by washing this extract (while still dissolved in methylene chloride) with buffers of increasing pH, the lipoic acid could be completely extracted into the water phase at pH 6.0. No stearic acid was found to extract into the water phase even at pH 9.2, the highest tested. Thus, the lipoic acid can be separated from the fatty acids by simply washing the methylene chloride extract with bicarbonate buffer.

Release of the lipoic acid from *E. coli* was found to be 95% complete after 2 h at 120 °C in 6 M HCl as evaluated by methylene chloride extraction and GC analysis of the lipoic acid as the *S,S*-dibenzyl methyl ester. The addition of several thiol protecting agents such as mercaptoacetic acid, mercaptoethanol, or ethanethiol to the hydrolysis mixture did not increase the yield. No improvement in the yield was noted when 6 N sulfuric acid was used for the hydrolysis.

On the basis of these findings, the standard assay procedure for lipoic acid analysis reported under Experimental Section was developed. Injection of 1/10 of the sample from 8 g wet weight of cells isolated by the above procedure gave a single GC peak equivalent to 2 μg of lipoic acid as the dibenzyl methyl ester. The mass spectrum of this peak (Figure 1a) was exactly the same as that prepared from a known sample of DL-lipoic acid. The important features of the mass spectrum for our discussion here are the strong ion at $M^+ - 91$, *m/e* 311, which contains all of the hydrogens of the lipoic acid and the weak ion at *m/e* 137 which results from cleavage of the $C_7$ and $C_8$ bond of the lipoic acid derivative. The *m/e* 137 ion is important because it contains the two hydrogens on $C_8$ of the original lipoic acid and, as such, can be used to measure the deuterium incorporated biosynthetically at this carbon. That both $C_8$ hydrogens are retained in this fragment was confirmed by the mass spectrum of methyl [6,8,8′-$^2H_3$]-6,8-bis([α,α′-$^2H_2$]benzylthio)octanoate. In this compound the *m/e* 137 ion was found to increase 4 mass units, indicating that the two deuteriums at $C_8$ are retained in the fragment. (Note the other two deuteriums are incorporated at the benzylic hydrogens during the exchange.)

When the cells are grown in the presence of 6 g/L of [*methyl*-$^2H_3$]acetate and the lipoic acid is extracted, derivatized, and assayed by GC–MS, the mass spectrum shown in Figure 1b is obtained. A large incorporation of deuterium is clearly evident. By subtraction of the natural isotopic abundances (determined experimentally for the *m/e* 311 ion on a nonlabeled sample) from the isotope peaks in the *m/e* 311 ion for the deuterated sample, the distribution of deuterium in the lipoic acid can be obtained. The results obtained for this sample, as well as for lipoic acid, from cells grown on 4 and 8 g/L of deuterated acetate are shown in Table I.

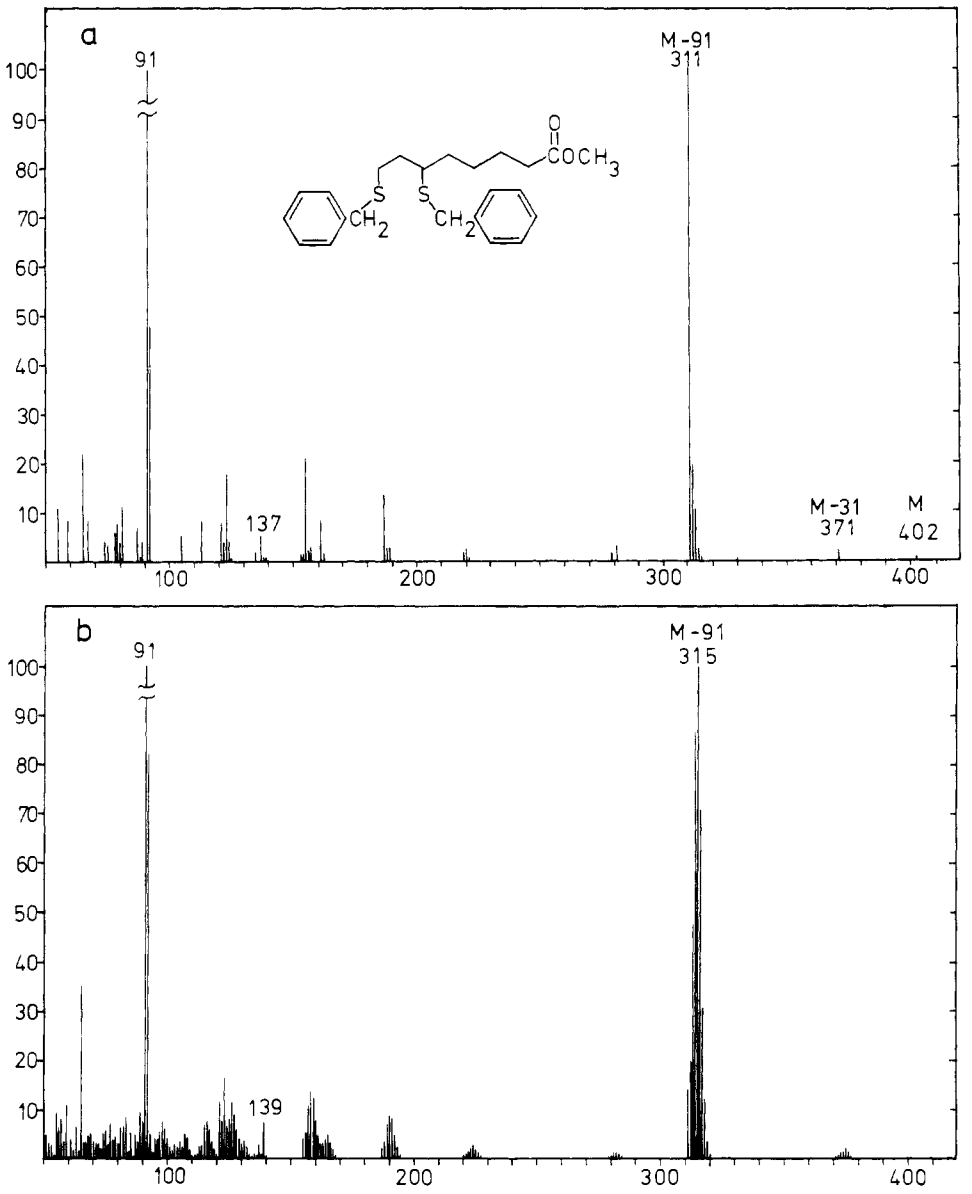


FIGURE 1: Mass spectrum of methyl 6,8-bis(benzylthio)octanoate. (a) Prepared from lipoic acid isolated from cells grown on unlabeled acetate; (b) prepared from lipoic acid isolated from cells grown in the presence of 6 g/L [*methyl*-$^2H_3$]acetate.

The observed incorporation of up to eight deuteriums into some of the lipoic acid is strong evidence that the lipoic acid is being biosynthesized from a fatty acid. Two deuteriums are likely to be located on $C_8$, and one deuterium should be present on each of the other carbons except $C_1$. As in the case of the long-chain fatty acids, we would expect different amounts of deuterium to be incorporated at the even and odd carbons and at the methyl.

An obvious choice for a fatty acid precursor would be octanoic acid. Analysis of the octanoic acid in the cells as the $Me_3Si$ derivative showed it to have the following relative intensities for the M − 15 ion at *m/e* 211: *m/e* 214, 48; *m/e* 215, 68; *m/e* 216, 100; *m/e* 217, 74; *m/e* 218, 30. (The ion intensities for the *m/e* 211 to *m/e* 213 ions and those higher than *m/e* 218 could not be measured accurately due to the very small amount of octanoic acid present.) These relative ion intensities were almost identical with those observed for the deuterated lipoic acid but were shifted up by 1 mass unit. This would indicate that, if the lipoic acid is derived from octanoic acid, then one highly deuterated hydrogen must be lost in the conversion. This clearly must come from the terminal methyl group since this is the only position of the biosynthesized fatty acids that is this extensively labeled, each methyl hydrogen having ~80% deuterium (White, 1980).

Confirmation that octanoic acid is a precursor was obtained by feeding [U-$^2H_{15}$]octanoate at 0.1 mg/mL to a growing *E. coli* culture. GC–MS of the isolated lipoic acid showed that 90.3% of the sample had been biosynthesized from the fed octanoate and that the labeled molecules contained 13 deuteriums. (This statement takes into account the 6.5% exchange at $C_2$ during the isolation of the lipoic acid and the 98 atom % deuterium in the original octanoic acid.)

This result clearly not only demonstrates that octanoic acid is a precursor of lipoic acid in *E. coli* but also shows that the sulfur atoms are inserted into the octanoic acid with the removal of only the replaced hydrogens.

Finally, it can be determined from the deuterium incorporation data whether or not the deuterium on the $C_6$ carbon of the octanoic acid, biosynthetically derived from acetate, is still present after conversion into lipoic acid. This information can be obtained by comparing the experimentally determined lipoic acid deuterium distributions with the calculated values. The calculated values are generated by eq 1 when the $C_6$ deuterium is retained and from eq 2 when the $C_6$ deuterium

$$(a + b)(c + d)^3(x + y)^3 \quad (1)$$

$$(a + b)(c + d)^2(x + y)^3 \quad (2)$$

is lost. [In these formulas, the ratio $b/(a + b)$ represents the fraction of the molecule with two deuteriums at $C_8$, the ratio $d/(c + d)$ represents the fraction of deuteration of the one hydrogen on the even carbons which is derived from acetate, and the ratio $y/(x + y)$ represents the fraction of deuteration of the one hydrogen on the odd carbons which is derived from acetate via NADPH.]

Since each term of the above equations can be independently evaluated, the correct formula can be determined by establishing which formula, expanded with the experimental $b$, $d$, and $y$ values, best describes the experimental results.

The value for $b$ can be confirmed from the incorporation of deuterium into the $m/e$ 137 fragment ion of the methyl 6,8-bis(benzylthio)octanoate. In the spectrum of this compound from the 6 g/L acetate growth, at least 70% of the $m/e$ 137 ion intensity was found to increase 2 mass units. This value for $b$ was also confirmed by the evaluation of the deuterium distribution in the fatty acids from the growth experiment which showed that 80% of the methyl groups contain three deuteriums. Values for $d$ and $y$ of 45 and 20%, respectively, were also established from this work (White, 1980).

The values of $y$ for each of the acetate feeding experiments were also determined from an analysis of the deuterium incorporated into threonine and proline from NADPH as discussed under Experimental Section. The values were found to be 18, 19, and 20% for the 4, 6, and 8 g/L acetate feedings, respectively. Even though the cellular NADH pools in each of these experiments had ~10% deuterium (see Experimental Section), these deuteriums are not incorporated into fatty acids and, therefore, do not affect the calculation.

It is clear from Table I that the best fit between the calculated and observed data occurs when the deuterium at $C_6$ is retained. Since it has been previously shown that the acetate-derived hydrogens on the even carbons are incorporated in an L configuration (White, 1980) and that the $C_6$ hydrogen in lipoic acid is in the D configuration (Mislow & Meluch, 1956), then the stereochemistry at $C_6$ of octanoate must be inverted during its conversion to lipoic acid. This result confirms the findings of Parry & Trainor (1977), who have shown, using stereospecifically tritiated octanoic acids, that the sulfur atom at $C_6$ is introduced with inversion of configuration.

## Discussion

On the basis of the above finding, a logical pathway for lipoic acid biosynthesis would involve hydroxylated octanoic acid intermediates. The strongest support for this pathway comes from the observed inversion of the stereochemistry at $C_6$ which occurs at some stage of the sulfur insertion. This can be most easily explained by the formation of a $C_6$ hydroxylated octanoic acid intermediate with a D stereochemistry, followed by the inversion of this stereochemistry upon sulfur insertion. The introduction of $C_8$ could be explained in the same manner. Both of these reactions would be analogous to the well-established conversion of serine to cysteine by sulfide. The formation of the $C_6$ and $C_8$ hydroxylated intermediate would involve an $\omega$- and an ($\omega$-2)-hydroxylation. Both of these hydroxylations are known to occur in bacteria and to require molecular oxygen (Kusunose et al., 1964; Miura & Fulco, 1974).

However, two additional experimental facts tend to discredit this explanation: first, the observation that 8-hydroxyoctanoic acid is not a precursor for lipoic acid in *E. coli*, and, second, *E. coli* grown anaerobically still are able to biosynthesize lipoic acid (R. H. White, unpublished data). These observations then call for the postulation of a sulfur insertion mechanism in which molecular oxygen is not required for the production of a necessary intermediate.

One possible mechanism which could account for the introduction of the sulfur would be a reaction analogous to that involved in the insertion of oxygen into hydrocarbons by monoxygenases, but using sulfur instead of oxygen. Since it is known that sulfur atoms can directly insert in C–H bonds of hydrocarbons (Knight et al., 1963), then an enzymatic system capable of generating "carbene"-type sulfur could perform a thiolation the same as oxygen can perform an hydroxylation in monooxygenases. If this is in fact the mechanism of sulfur insertion, then unlike most hydroxylations, which proceed with retention of configuration, this thiolation must proceed with inversion of configuration. Since there are no known examples of this type of enzymatic reaction, this cannot be used as an argument for or against this proposed mechanism.

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