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Stable Isotope Studies on the Biosynthesis of the Thiazole Moiety of Thiamin in *Escherichia coli*†

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ABSTRACT: Deuterated and ^{13}C-labeled sugars were fed to *Escherichia coli* growing on defined medium. The position and extent of incorporation of the label into the 4-methyl-5-β-hydroxyethylthiazole portion of thiamin and other cellular components were measured by gas chromatography–mass spectrometry. Based on the findings, it is concluded that the contiguous five-carbon unit of the 4-methyl-5-β-hydroxyethylthiazole is biosynthetically derived from pyruvate and a triose phosphate.

Most previous studies concerning biosynthetic pathways in microorganisms have involved the feeding of small quantities of radioactive precursors followed by the laborious isolation and chemical degradation of the product to determine both the extent of incorporation and the position of the incorporated label. This approach has several limitations which can restrict its use with intact microorganisms. These include: inability of the precursor to permeate the cell; feeding of the incorrect form of the precursor for metabolism to the desired compound, i.e., not phosphorylated; and metabolism of the compound to a primary metabolite, i.e., acetate, which often can be incorporated nonspecifically back into the product of interest.

These problems may be avoided by growing the organism

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on a medium where a large portion of the metabolized carbon is from a carbon source specifically labeled with a stable isotope. The cell would then produce labeled natural precursors which are biosynthetically incorporated into the compound under study. Comparison of the position and extent of label incorporation into this compound and other cellular constituents can then supply information as to the nature of its biosynthetic pathway. In many cases, evaluating the extent and position of label incorporation is best determined by mass spectrometry.

The use of this approach in determining the biosynthetic origins of the carbons and hydrogens of the 4-methyl-5-β-hydroxyethylthiazole portion of thiamin is illustrated in this report. The feeding of stable isotope-labeled compounds to *Escherichia coli* and measuring both the position and extent of incorporation of the labels into the thiazole and other cellular constituents are described. By comparison of the distribution of the label in these products, it is concluded that the five-carbon unit of the thiazole is derived from pyruvate and a triose phosphate. Since previous work (White and Rudolph, 1978; Estramareix and Therisod, 1972; Bellion et al., 1976) has shown the nitrogen and the thiazole C-2 to be derived from tyrosine, this leaves only the biosynthetic origin of the sulfur in doubt.

Experimental Section

Materials

D-[U-^{13}C]Glucose (88.1 atom % ^{13}C) was obtained from Merck, Sharp and Dohme of Canada. The sample was diluted with 9 parts of unlabeled D-glucose before feeding. Sodium borodeuteride, 99% ^{2}H, was obtained from Stohler Chemical Co. D-Glyceraldehyde, L-glyceraldehyde, dihydroxyacetone, pyruvic acid, deuterium oxide, D-glucuronic acid lactone, and wheat germ acid phosphatase were obtained from Sigma Chemical Co. [*Methyl*-$^{2}H_3$]acetic acid, 99.5% ^{2}H, and 4-methyl-5-β-hydroxyethylthiazole were obtained from Aldrich Chemical Co. Casamino acids were obtained from Difco Laboratories and contained 10% nitrogen.

Methods

Maintenance and Growth of the Organism. E. coli B was grown on 100 mL of defined liquid medium at 37 °C in a 2-L wide-mouthed Erlenmeyer flask on a rotary shaker. The medium consisted of 12.1 g of Tris,[1] 9.9 g of K_2HPO_4, 5.3 g of NH_4Cl, 5 g of casamino acids, 2.9 g of sodium citrate·$2H_2O$, 4 g of the labeled compound, 0.5 g of NaCl, 120 mg of $MgSO_4$, 44 mg of $CaCl_2$·$2H_2O$, and 0.8 mg of $FeCl_3$·$6H_2O$ per L of water. The pH was adjusted to 7.0 with HCl. Stock cultures were maintained on agar slants using this same medium but containing the appropriate nonlabeled sugar. Organisms from 24-h-old slants grown at 37 °C containing the same carbon source as the growth medium were used to inoculate the growth medium which was then allowed to grow to the end of log phase (10–12 h).

Standard Method for the Isolation and GC–MS Analysis of 4-Methyl-5-β-hydroxyethylthiazole. Fresh *E. coli* cells (1–2 g) isolated at the end of log phase growth were separated from the growth medium by centrifugation (5000*g*, 10 min) and resuspended in 8 mL of 0.1 M HCl. The cells were then placed in a boiling water bath for 20 min to extract the thiamin, after which they were cooled to room temperature, 1 mL of 1 M sodium acetate (pH 4.7) was added, and the pH was adjusted to 4.7 with 2 M KOH. Centrifugation of this material at 30 000*g* for 20 min gave a clear yellow supernatant which was removed from the cell pellet and mixed with 20 mg of wheat germ acid phosphatase; 2 drops of toluene was added to inhibit bacterial growth. After incubation at 37 °C for 18 h, 1.2 g of solid sodium bisulfite was added and the incubation was continued another 14–18 h. At the end of this time, the tubes were cooled to 0 °C and 4 mL of 50% NaOH was added. The contents were then extracted twice with ~6 mL of methylene chloride. The combined extracts were evaporated under a stream of nitrogen in a water bath at 37 °C. The residue containing the isolated 4-methyl-5-β-hydroxyethylthiazole was dissolved in 30 μL of trifluoroacetic anhydride/methylene chloride (1:1) prior to GC–MS analysis.

Amino Acid Isolation and Derivation. A portion (~100 mg) of the insoluble cell pellet obtained in the above procedure was washed with 0.1 M HCl and hydrolyzed with 2 mL of 6 M HCl at 110 °C for 24 h. The resulting sample was filtered, evaporated to dryness, dissolved in a small volume of water, and applied to a small column (4 × 40 mm) of Dowex 50W-X8 (H^+), 50–100 mesh. After washing with 10 column volumes of water, the amino acids were eluted with 3 M aqueous ammonia. Following evaporation of the solvent, the dried sample of amino acids was heated for 3 h at 110 °C with 3 mL of 3 M HCl in 1-butanol. After removal of the solvent, the resulting butyl esters were converted to their *N*- and *O*-trifluoroacetates by reaction with 50% trifluoroacetic anhydride in methylene chloride for 12 h at room temperature. The solvent was then removed, and the residue taken up in methylene chloride for GC–MS analysis. Known samples were prepared in an identical manner.

Preparation of Trimethylsilyl Derivatives. Trimethylsilyl ethers were prepared by reacting ~2 mg of the material to be derivatized with 0.1 mL of a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (9:3:1). After 1 h at 60 °C, the resulting solution was assayed directly by GC–MS.

Glycerol Phosphate Isolation and Isotopic Analysis. A portion (~300 mg) of the insoluble cell pellet obtained from the thiamin extraction was extracted with 20 volumes of methylene chloride–methanol (1:1) and filtered to remove insoluble material. Water (20 volumes) was added and, after shaking, the lipid was recovered by evaporation of the methylene chloride layer. This material was then mixed with 1 mL of 2 M HCl and 2 mL of methanol and hydrolyzed at 110 °C for 20 h. The resulting hydrolysate was extracted with methylene chloride to remove the fatty acids; the glycerol phosphate was isolated by evaporation of the water layer. After conversion to the $(Me_3Si)_4$ ether, the glycerol phosphate was assayed by GC–MS to determine its deuterium content. GC–MS of each sample assayed gave two peaks representing ~85% α-glycerol phosphate and ~15% β-glycerol phosphate. The deuterium content of each peak was measured using the $M^+ - 15$ ion at *m/e* 445. The deuterium content of the $M^+ - (CH_2OMe_3Si)$ ion at *m/e* 357 was used to determine if the deuterium was in an α or β position. Hydrolysis and analysis of a known sample of DL-[1-^{2}H]glycerol 3-phosphate (prepared by reduction of DL-glyceraldehyde 3-phosphate with sodium borodeuteride) under the same conditions as used for the assayed samples showed no loss of deuterium. As expected, the phosphate group was found to randomize between C-1 and C-3 of the glycerol under acid hydrolysis, thus making it impossible to determine on which of these carbons the original deuterium resided.

Gas Chromatography–Mass Spectrometry. An LKB 9000 S gas chromatograph–mass spectrometer equipped with a 6 ft × 1/8 in. column packed with 3% OV-17 on Gas-Chrom Q

[1] Abbreviation used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

(Applied Science, Inc.) was used for the analyses described herein. 4-Methyl-5-β-hydroxyethylthiazole trifluoroacetate was separated with a column temperature of 125 °C and an injection temperature of 200 °C. The Me_3Si derivative of glycerol phosphate was assayed with a column temperature of 180 °C and an injection temperature of 250 °C. Under these conditions, the α-glycerol derivative had a retention time of 3.7 min and the β-glycerol derivative had a retention time of 4.3 min. The amino acids were separated by temperature programming from 60 to 270 °C at 10 °C/min with the injection port maintained at 280 °C. All spectra were recorded at 70 eV with an ion source temperature of 270 °C and a separator temperature of 300 °C. The level of incorporation of label into the 4-methyl-5-β-hydroxyethylthiazole trifluoroacetates, the Me_3Si carbohydrates, and the amino acids was calculated from the mass spectra obtained by repeated scanning (six to eight times) of the appropriate peak as it eluted from the gas chromatograph. The ion intensity for each major ion of interest in each scan was then measured and summed. The same process was also performed on the associated isotope peaks. The normalized abundances of the isotopic peaks were then calculated from this data. This method of summing the ion intensities over the entire GC peak was required because of the isotope fractionation which occurred during the GC of the deuterated compounds (Wilzbach and Riesz, 1957). Ion intensities could be determined to within ±1% from the oscillographic traces. For each analysis performed on a labeled molecule an identical analysis at the same concentration was performed on a nonlabeled sample. The percent of isotopic enrichment was then calculated from these two sets of data as outlined by Biemann (1962).

Preparation of 4-Methyl-5-[β-$^2H_2$]-β-hydroxyethylthiazole. 4-Methyl-5-β-hydroxyethylthiazole (286 mg) was oxidized by 474 mg of potassium permanganate in 6 mL of 1 M HCl at room temperature for 10 min. After treatment with sodium bisulfite to dissolve the precipitated manganese dioxide, the reaction mixture was applied to a small column of Dowex 5W-X8 (H^+). After washing with 10 column volumes of water, the 4-methylthiazole-5-acetic acid (100 mg) was eluted with 3 M ammonia. Following evaporation of the solvent, the ammonium salt of the acid was converted to the methyl ester (M^+, *m/e* 271) by reaction with anhydrous HCl in methanol as described by Cerecedo and Tolbin (1937). The ester was reduced to the β-dideuterated alcohol by reduction with lithium aluminum deuteride in ether. The final compound contained 98% $^2H_2$ as determined by the mass spectrum of its trifluoroacetate ester.

Preparation of 1,2-O-Isopropylidene-α-D-[6,6′-$^2H_2$]-glucofuranose. To a solution of 4.0 g of 1,2-*O*-isopropylideneglucofuranuronolactone [prepared as described by Owen et al. (1941)] in 20 mL of water cooled to 0 °C was added 480 mg of sodium borodeuteride over 10 min with constant stirring. After sitting overnight at 3 °C, the resulting solution was passed through a Dowex 50 H^+ column and concentrated to dryness in a vacuum at 50 °C. The residue was dissolved three times in 100 mL of methanol and evaporated after each methanol addition to remove the borate. Gas chromatography of this material as the Me_3Si derivative showed 77% conversion to the desired compound with 18% being the open lactone of the starting sugar and 4% unreacted starting lactone. After four recrystallizations from ethyl acetate, 2.85 g of a white solid was isolated with mp 159–160 °C. This agrees with the previously reported value of 158–160 °C given by Mackie and Perlin (1965). GC–MS of this material gave a single GC peak as the $(Me_3Si)_3$ derivative and a mass spectrum showing a $M^+ - 15$ at *m/e* 423 showing the molecule to have 96.5% $^2H_2$. The location of the two deuterium atoms at C-6 was confirmed by the absence of deuterium in the *m/e* 333 fragment ($M^+ - C^2H_2OMe_3Si$).

Preparation of 1,2-O-Isopropylidene-α-D-[5,6,6′-$^2H_2$]-glucofuranose. The 5-keto derivative of 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone was prepared from the 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone 5-nitrate by base elimination of nitrite ion as described by Dax and Weidmann (1972). The resulting keto compound was reduced with sodium borohydride as described by Mackie and Perlin (1965). The final product, after three recrystallizations from ethyl acetate, had a mp of 158–159 °C and gave a single GC peak as the $(Me_3Si)_3$ derivative. Mass spectrum of this peak showed a $M^+ - 15$ fragment at *m/e* 424 containing 93.5% $^2H_3$. The fragment at *m/e* 334 ($M^+ - C^2H_2OMe_3Si$) confirmed that two deuterium atoms were on C-6.

Hydrolysis of Sugar Derivatives. Both of the above deuterated glucose derivatives were quantitatively converted to an equilibrium mixture of α- and β-D-glucose by hydrolysis in 0.1 M HCl at 80 °C for 1 h. Evaporation in a desiccator over sodium hydroxide was used to remove the acetone and HCl. GC–MS of the isolated sugars as the Me_3Si derivatives confirmed that no loss of deuterium had occurred during the hydrolysis.

Preparation of [Methyl-$^2H_3$]pyruvate. Pyruvic acid (2 g) was dissolved in 10 g of deuterium oxide and heated at 100 °C for 2 h. After cooling, 0.5 mL of H_2SO_4 was added and the water layer extracted seven times with 3 mL of ether. The combined ether layers were dried with anhydrous sodium sulfate and evaporated to give 1 g of yellow oil which was subsequently purified by vacuum sublimation. The purity of the sample and the deuterium content of the methyl group were measured by reduction with sodium borohydride in aqueous solution followed by GC–MS of the resulting lactic acid as the $(Me_3Si)_2$ derivative. Measurements of the $M^+ - 15$ ion at *m/e* 229 were used to calculate the following distribution of deuterium in the pyruvate methyl group: 3.3% $^2H_0$, 3.5% $^2H_1$, 26% $^2H_2$, and 70% $^2H_3$. Only one GC peak was observed with the same retention time as authentic lactic acid bis(trimethylsilane).

Preparation of L-[1-^2H]-, [2-^2H]-, and L-[3-^2H]Glycerol. The monodeuterated glycerols were prepared by the sodium borohydride (120 mg) reduction of 1-g samples of L-glyceraldehyde, dihydroxyacetone, and D-glyceraldehyde dissolved in 5 mL of H_2O at 0 °C for 12 h. After reaction, the samples were desalted by passage through a Dowex 50 H^+ column and the borate was removed by evaporation with methanol. The resulting clear, colorless syrups were shown to contain 99% $^2H_1$ by GC–MS of their $(Me_3Si)_3$ derivatives.

Results and Discussion

Before incorporation of stable isotopes into the thiazole moiety of thiamin can be determined, a complete understanding of the fragmentation pattern of its trifluoroacetate must be established. Figure 1 shows the low-resolution spectrum, as determined by GC–MS of a 3.5-μg sample of 4-methyl-5-β-hydroxyethylthiazole trifluoroacetate. A molecular ion (M^+) is at *m/e* 239 and intense fragment ions are at *m/e* 112 and 125. The postulated origins of these and other ions in the spectrum are outlined in Figure 3. The *m/e* 125 ion results from the elimination of CF_3COOH from the molecular ion with the proton of the eliminated fragment coming from C-7. (See Figure 3 for the numbering system used in this paper for the substituted thiazole ring.) A decrease in the amount of deuterium in the *m/e* 125 ion as compared to the molecular ion reflects the incorporation of a deuterium at C-7. If only one

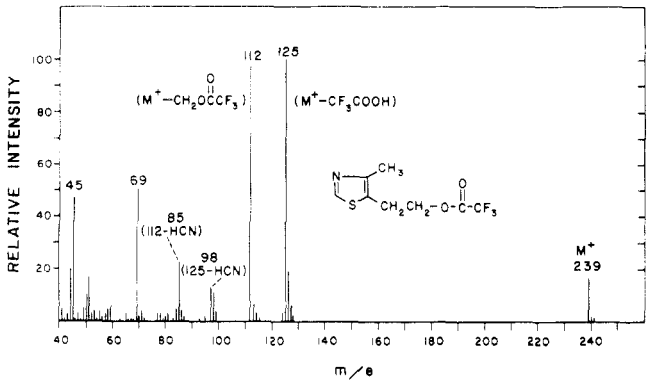

FIGURE 1: Mass spectrum of 4-methyl-5-β-hydroxyethylthiazole trifluoroacetate. The mass spectrum was obtained from 1.8 μg of 4-methyl-5-β-hydroxyethylthiazole isolated from 160 mg of *E. coli* cells by the methods outlined in the text.

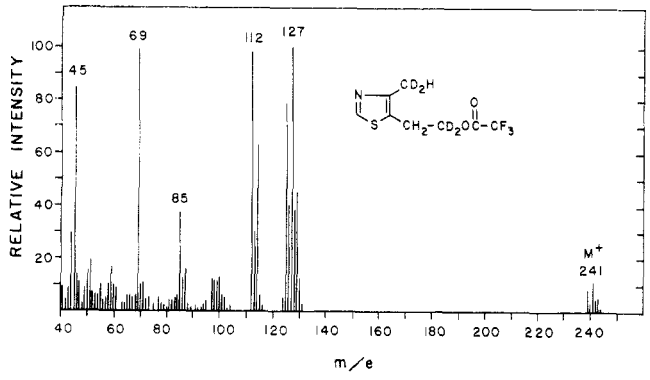

FIGURE 2: Mass spectrum of 4-methyl-5-β-hydroxyethylthiazole trifluoroacetate isolated from cells grown in the presence of D-[6,6′-$^2H_2$]-glucose.

deuterium is incorporated at C-7, then we would expect to see only a 50% loss of this deuterium because of competition from the loss of the equivalent proton on C-7. This is assuming that there is no deuterium isotope effect on the elimination reaction.

The *m/e* 112 ion results from the simple cleavage of the bond between C-7 and C-8 with the positive charge being retained on the thiazole fragment. This fragment retains the protons of the C-6 methyl as well as the C-7 protons. If the original molecule has no deuterium at C-7, then the deuterium content of the *m/e* 112 ion will reflect the incorporation of deuterium in the methyl group. The difference between the deuterium content of the M^+ *m/e* 239 or 125 ions and the *m/e* 112 ion will allow calculation of the deuterium incorporated at C-8. That the fragmentation of the C-7–C-8 bond can be used in this manner to calculate the amount and position of the deuterium is confirmed by the mass spectra of 4-methyl-5-[β-$^2H_2$]-β-hydroxyethylthiazole trifluoroacetate. In this compound, the *m/e* 112 ion is still present and contains no deuterium, whereas both the molecular ions at *m/e* 239 and 125 have increased 2 *m/e*. In addition, the *m/e* 127 ion still retains both of the deuteriums which eliminates the C-8 protons as the origin of protons for the eliminated CF_3COOH in the production of the *m/e* 125 ion.

Incorporation of deuterium at C-2 need not be considered, since Breslow (1957) has demonstrated that the aromatic hydrogen at the C-2 position of the thiazolium ring of thiamin exchanges readily with water.

Having established how the mass spectral data can be evaluated, the label distribution in biosynthetically produced

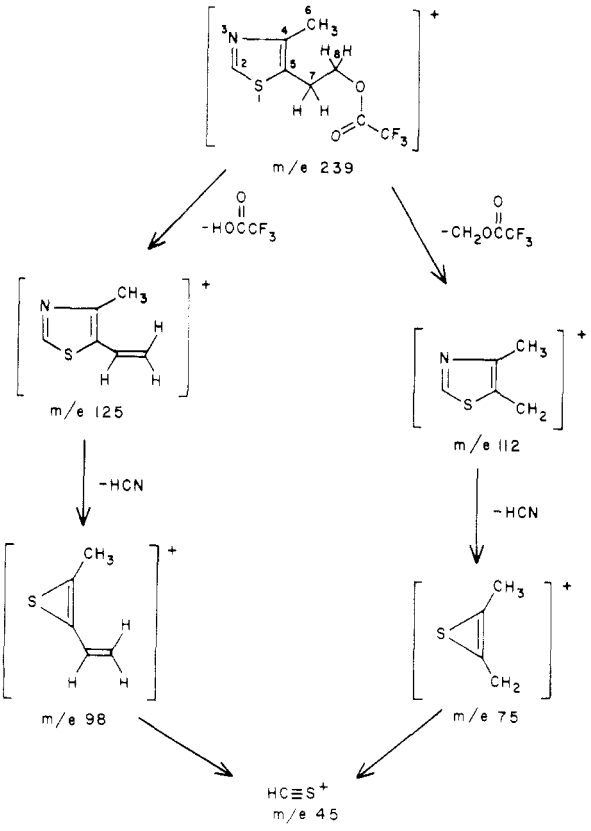

FIGURE 3: Fragmentation of 4-methyl-5-β-hydroxyethylthiazole trifluoroacetate.

TABLE I: Incorporation of ^2H- and ^{13}C-Labeled Glucose into 4-Methyl-5-β-hydroxyethylthiazole by *E. coli*.[a]

labeled Glc fed	no. of ^2H	incorp. (% of total) M^+	125	112
D-[6,6′-$^2H_2$]Glc	0	33.8	33.5	62
	1	8.9	9.2	11.5
	2	38.2	36.7	25.7
	3	6.4	7.0	<0.5
	4	12.5	13.0	<0.5
D-[5,6,6′-$^2H_3$]Glc	0	33.0	33.0	53.0
	1	10.8	10.5	19.0
	2	31.2	32.4	22.7
	3	13.2	10.6	5.4
	4	7.4	11.5	<0.5
	5	4.0	2.0	<0.5
	no. of ^{13}C	incorp. (% of total) M^+	125	112
D-[U-^{13}C]Glc	0	74.7	73.8	77.6
	1	12.3	11.1	9.6
	2	7.5	8.3	11.9
	3	5.4	5.8	0.50
	4	0.9	1.0	0.52

[a] Data are presented as the percent of the total number of molecules or ions containing the indicated number of isotopes. The [^{13}C]glucose contained only 10% of the molecules with D-[U-^{13}C]glucose (88.1 atom % ^{13}C).

thiazoles can be studied. Table I summarizes the observed incorporation of deuterium and ^{13}C into 4-methyl-5-β-hydroxyethylthiazole by *E. coli* grown in the presence of labeled glucoses. Figure 2 shows a representative mass spectrum of the

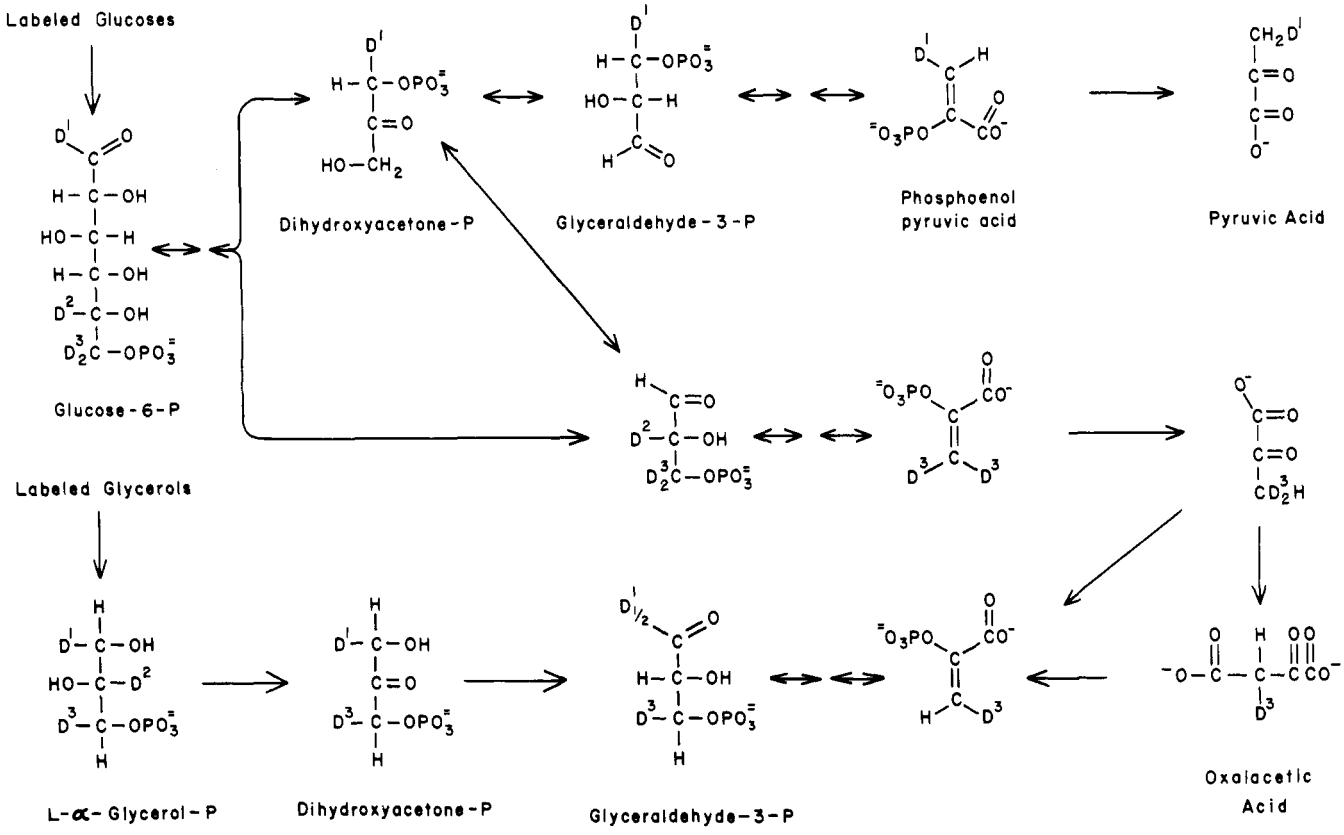

FIGURE 4: Metabolism of deuterium-labeled glucoses and glycerols by *E. coli*.

thiazole isolated from the cells grown on D-[6,6′-$^2H_2$]glucose. The results for this D-[6,6′-$^2H_2$]glucose feeding clearly indicate that the thiazole C-6 and C-8 protons and their attached carbons have their origins from the C-6 of glucose. Since the isotope compositions of the M^+ and *m/e* 125 ions are about the same, no deuterium is located on the thiazole C-7. These data strongly support the idea that the five-carbon unit of the thiazole must be derived from at least two fragments of glucose in such a way that most of the deuterium in both fragments is retained. Considering the distribution of label in the *m/e* 112 ion, we find that 37.2% of the methyl groups is derived from the C-6 of glucose with 11.5% of these having been synthesized from a fragment containing only one proton. This one proton fragment can be generated by the resynthesis of a small amount of the glucose-derived pyruvate containing only one deuterium. This could occur either indirectly via oxaloacetate and phosphoenolpyruvate or more directly via phosphoenolpyruvate as outlined in Figure 4. In any case, for each pyruvate resynthesized approximately 66% will retain only one deuterium.

In contrast to the isotopic distribution found for the methyl group, the deuterium distribution for the thiazole C-8 is found to consist of 45% with two deuteriums and 5% with one deuterium. (These values are obtained from the difference in the isotopic distribution of the *m/e* 112 ion and that observed for the entire molecule.) This reduced occurrence of C-8 with one deuterium as well as the increased incorporation of carbons with two deuteriums argues that the biosynthetic fragment which gives the thiazole C-8 has its origin closer to the glucose and that giving rise to the methyl fragment closer to pyruvate.

The lower content of single deuterium-containing fragments in metabolites closer to glucose is confirmed by the analysis of the glycerol phosphates from these cells. This analysis showed 35.4% of the molecules to have two deuteriums and 5% one deuterium. The lower overall content of deuterium in the glycerol phosphate than that observed in the thiazole C-8 can be rationalized by the lack of complete equilibration of the glucose-derived deuterated glyceraldehyde 3-phosphate with dihydroxyacetone before it is reduced by the cell to glycerol phosphate.

If the C-6 glucose protons are incorporated into the thiazole, then we would expect the C-1 proton to be incorporated. Feeding of D-[1-^{2}H]glucose showed the thiazole to have 22% with one deuterium and 2.1% with two deuterium, which reconfirms that the molecule is constructed from two parts. The low incorporation of label from the D-[1-^{2}H]glucose must result from the metabolism of a large part of the glucose via the glucose 6-phosphate oxidative-pentose phosphate pathway. Metabolism of glucose by this pathway would result in the complete loss of the C-1 deuterium. In contrast, the glucose C-6 protons would not be lost and would eventually be metabolized (as outlined in Figure 4) after recycling back to glucose 6-phosphate.

A logical extension of the above conclusions is that the thiazole C-4 and C-7 originate from the C-5 of glucose. This was confirmed for the thiazole C-7 by feeding glucose labeled at both C-6 and C-5 with deuterium. That a portion of the C-5 glucose is incorporated in the thiazole C-7 is shown by the data reported in Table I in which a small fraction of the molecules contain five deuterium atoms. That the additional deuterium is incorporated at C-7 is indicated by the loss of one-half of the molecules containing five deuterium in the *m/e* 125 ion. This loss is also confirmed by a decrease in the number of *m/e* 125 ions with three deuterium atoms as compared to the molecular ion. The low incorporation of the C-5 deuterium of glucose can be accounted for by the loss of this deuterium from the glucose-derived glyceraldehyde 3-phosphate by equilibration with dihydroxyacetone phosphate catalyzed by triose-phosphate isomerase.

TABLE II: Incorporation of Monodeuterated Glycerols into 4-Methyl-5-β-hydroxyethylthiazole and Glycerol Phosphate by *E. coli*.

fed glycerol	no. of ^2H	4-methyl-5-β-hydroxyethylthiazole incorp. (% of total) M$^+$	125	112	glycerol-P
L-[1-^{2}H]glycerol	0	91.5	91.5	95.5	>95% $^2H_1$ on α-C
	1	8.5	8.5	4.5	
	2	0	0	0	
[2-^{2}H]glycerol			less 0.2% any ion		>95% $^2H_1$ on β-C
L-[3-^{2}H]glycerol	0	26.2	26.3	61.0	>95% $^2H_1$ on α-C
	1	45.3	45.4	38.4	
	2	28.1	28.0	0	

TABLE III: Incorporation of [*Methyl*-$^2H_3$]acetate and [*Methyl*-$^2H_3$]pyruvate into 4-Methyl-5-β-hydroxyethylthiazole by *E. coli*.

labeled compd fed	no. of ^2H	incorp. (% of total) *m/e* 239	*m/e* 125	*m/e* 112
[*Me*-$^2H_3$]OAc	0	71.4	69.0	85.0
	1	25.6	28.1	13.4
	2	3.2	2.8	<0.5
[*Me*-$^2H_3$]Pyr	0	23.9	23.1	34.2
	1	15.6	17.1	17.0
	2	20.3	19.9	23.6
	3	24.7	23.8	25.3
	4	11.4	11.3	0
	5	4.1	4.9	0

The above deuterium data leave only the origins of the thiazole C-4 and C-5 atoms from glucose still in question. Since these carbons have no attached protons, ^{13}C must be used to determine their origin from glucose. The deuterium data indicate that the thiazole C-4 may come from the glucose C-5 and the thiazole C-5 from the glucose C-4. This can be confirmed by growing the *E. coli* on medium containing glucose, a small fraction of which has been uniformly labeled with ^{13}C. If the thiazole C-4 and C-6 come from glucose as an intact unit, then a portion of the molecules should have two ^{13}C atoms. Similarly, if the thiazole C-5 comes from the glucose C-4 intact with the C-5 and C-6 atoms, then we should see a portion of the molecules with three ^{13}C atoms. That this does occur is confirmed by the data reported in Table I. From the *m/e* 125 ion, we see that 5.8% of the thiazole molecules have three adjacent ^{13}C atoms and 8.3% have two adjacent ^{13}C atoms. Considering that the original glucose contained only 10% glucose uniformly labeled with 88.1% ^{13}C, then it can be calculated that 77.6% of a continuous three-carbon unit and 70.1% of a continuous two-carbon unit of the fed glucose are incorporated into the thiazole.

In addition, the *m/e* 112 fragment contains no significant amount of $^{13}C_3$ units, demonstrating that the C_3 unit must be carbons 5, 7, and 8 of the thiazole and the C_2 unit must be carbons 4 and 6.

From the ^{13}C incorporation data, we see that ~22% of the C_3 unit and 30% of the C_2 unit are not synthesized from the glucose carbons but come from the amino acids and/or citrate in the growth medium. Since the carbon atoms from these metabolites are most likely to enter gluconeogenesis as a three-carbon unit, i.e., PEP or pyruvate, then these data are consistent with the C_2 unit originating from a metabolite closer to PEP, and the C_3 unit from a metabolite closer to glucose.

As shown from the ^{13}C data in Table I, all the ions reported contain single ^{13}C atoms in excess of those calculated to occur in the C_2 and C_3 incorporated units. This increase is a result of the incorporation of ^{13}C into the thiazole C-2 and the incorporation of C_2 and C_3 units which have been resynthesized with only one ^{13}C. This resynthesis results from the condensation of $^{13}C_2$ acetyl CoA units with unlabeled oxalacetate which comes from either the unlabeled citrate or the unlabeled aspartic acid in the medium, or both. After one rotation of the citrate acid cycle, the resulting singly ^{13}C labeled malate or oxalacetate can be converted via phosphoenolpyruvate to C_2 and C_3 units containing only one ^{13}C. That these one ^{13}C-containing units were produced was confirmed by the observed incorporation of a slight excess of single ^{13}C atoms into amino acids which are biosynthesized from C_2 and C_3 units, i.e., alanine, phenylalanine, and tyrosine.

Considering the metabolic route of glucose metabolism in *E. coli*, it is very likely that the three-carbon fragment used in thiazole biosynthesis is a phosphorylated triose. This is also consistent with the fact that thiazole phosphate is the required substrate of thiamine-phosphate synthase in yeasts (Camiener and Brown, 1960).

Since it is well established that glycerol is metabolized in *E. coli* through α-D-glycerol phosphate as outlined in Figure 4 (Hayaski and Lin, 1965), it would be expected that labeled glycerols would likewise be incorporated into the thiazole. (The position of the deuterium in the glycerols is numbered according to the carbon atom to which it would be attached after conversion to L-α-glycerol phosphate as outlined in Figure 4.) In addition, which of the carbons of the glycerol become the thiazole C-5 may be determined by the loss or retention of label when the stereospecifically labeled glycerol is fed.

Table II shows the results of the deuterated glycerol feedings. We find that for the three different glycerols fed, only the L-[3-^{2}H]glycerol shows a significant incorporation. The distribution of deuterium in the thiazole sample is similar to that observed for the deuterated glucoses and reflects the synthesis from two units retaining one deuterium. The fact that no incorporation was observed with the [2-^{2}H]glycerol confirms that this proton is lost during glycerol metabolism by *E. coli*, as outlined in Figure 4.

Since the cellular glycerol still retains >95% of the molecules with one deuterium at C-2, then this eliminates glycerol phosphate as a direct precursor in the biosynthesis of the thiazole. In addition, the lack of incorporation of L-[1-^{2}H]glycerol supports the idea that the glycerol C-3 carbon is incorporated into the nonproton-containing C-5 thiazole carbon.

The above glucose and glycerol data indicate two possible origins of the two-carbon unit, acetate or an active form of acetate derived from pyruvate. The major requirement is that the methyl carbon protons must be incorporated without loss of deuterium. The true origin of the two-carbon unit can be determined by growth on the appropriate deuterated substrate. From the data presented in Table III, it is clear that the methyl group is not derived intact from acetate. Only one proton is

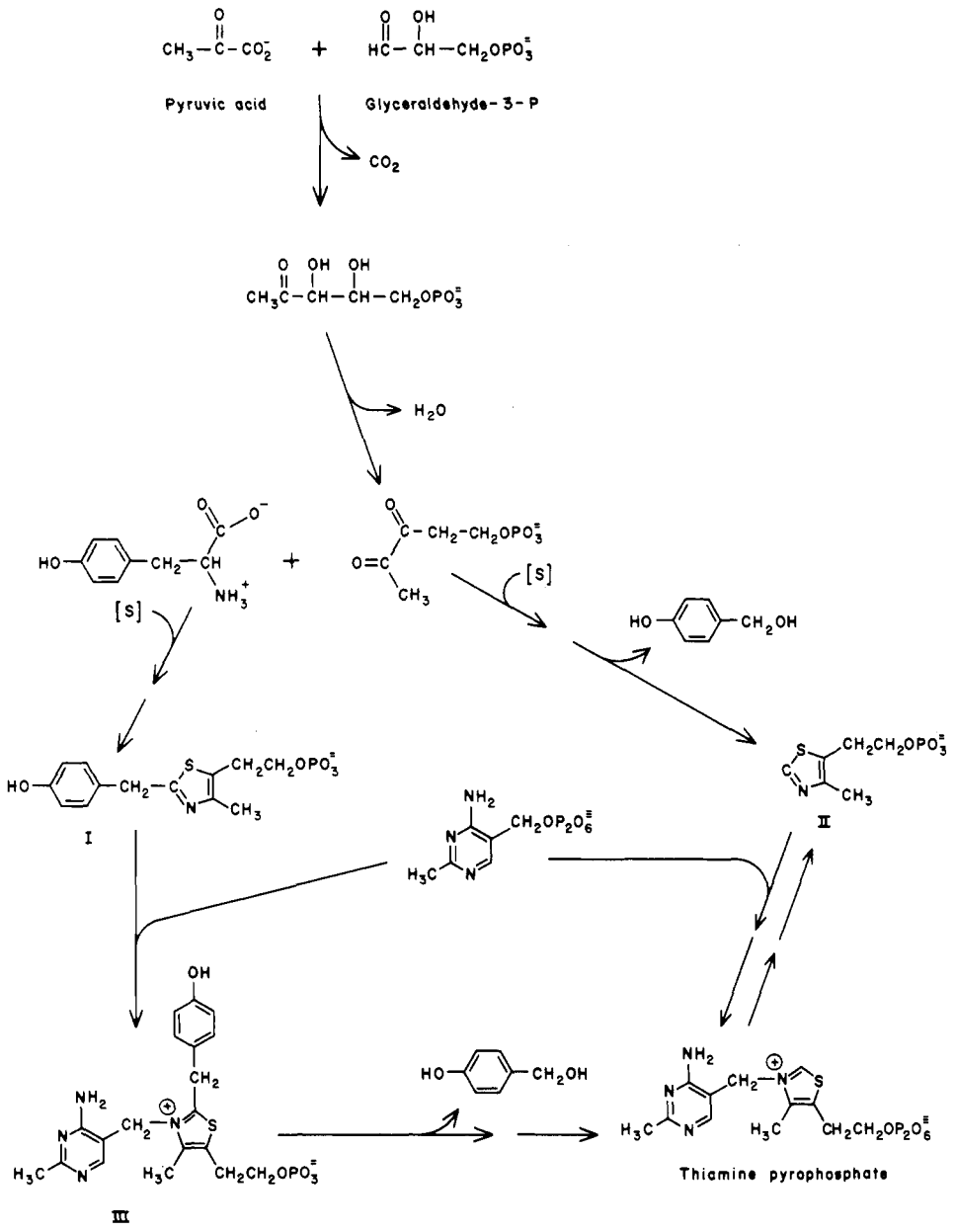

FIGURE 5: Proposed biosynthesis of 4-methyl-5-β-hydroxyethylthiazole in *E. coli*.

incorporated from acetate which appears to nonspecifically label all positions. However, for the pyruvate data we find that 25% of the molecules have a completely deuterated methyl group. This indicates that the methyl group of pyruvate goes into the thiazole as a complete unit. In addition, we find approximately 5% of the molecules to have five deuterium, which indicates that a portion of the triose phosphate has been synthesized via gluconeogenesis with two deuterium atoms.

Pyruvate incorporation into the thiazole is actually better than that indicated by the data shown in Table III. The reason for the lower observed incorporation is attributable to the fact that the pyruvate fed contained only 70% of the molecules with three deuterium atoms and that the carbon–deuterium bond in pyruvate exchanges spontaneously with the water. Trideuterated pyruvate was found to be chemically stable and to lose 20% of its deuterium in a sterile control experiment conducted under the same conditions as the pyruvate feeding.

The loss of isotope intercellularly during growth can be evaluated by isotope analysis of the cellular amino acids which are derived from pyruvate. The simplest of these, alanine, was found to have 59.7% no deuterium, 12.5% $^2H_1$, 13.8% $^2H_2$, and 14.1% $^2H_3$. As expected, the largest amount of the cellular alanine was incorporated from the medium and contained no label. However, for the alanine synthesized by the cell the ratios of molecules containing one, two, and three deuteriums are about the same as observed for the thiazole *m/e* 112 ion. Thus, the deuterium atoms incorporated into the alanine confirm that the deuterium content of the cellular pyruvate was indeed reduced to that observed in the thiazole methyl group. This isotopic dilution was also confirmed by the deuterium content of the cellular valine which showed 64.0% $^2H_0$, 4.7% $^2H_1$, 6.4% $^2H_2$, 6.5% $^2H_3$, 7.5% $^2H_4$, 6.7% $^2H_5$, and 4.5% $^2H_6$. The leucine and isoleucine showed a similar distribution.

Figure 5 outlines two possible pathways for the biosynthesis of the thiazole moiety of thiamin based on previously reported work as well as results presented here. The first step in each pathway involves an acyloin-type condensation between an activated two carbon of pyruvate with glyceraldehyde 3-phosphate to give the continuous five-carbon unit of the thiazole. This five-carbon unit is then condensed in some, as yet

undefined, series of reactions with the nitrogen and C-2 of tyrosine and a sulfur to form the thiazole ring. These reactions then yield either the unsubstituted 4-methyl-5-β-hydroxyethylthiazole phosphate derivative II or the *p*-hydroxybenzyl-substituted thiazole derivative I. Either of these products could give rise to thiamine diphosphate after condensation with 2-methyl-4-amino-5-hydroxymethylpyrimidine diphosphate as outlined in Figure 5. Although it has not yet been established which of these pathways is followed in *E. coli*, it has been confirmed that the *p*-hydroxybenzyl side chain of the original tyrosine is released during the thiazole biosynthesis as *p*-hydroxybenzyl alcohol (White, R. H., 1978, unpublished data). This finding places several chemical restraints on the specific chemical transformations allowed in the biosynthesis.

Available evidence indicates the occurrence of both of these pathways in nature. Nutritional and genetic studies on thiamin biosynthesis in mutants of *Neurospora* (Tatum and Bell, 1946; Harris, 1955) suggest at least two separate pathways for thiamin biosynthesis. In these studies, the major pathway was postulated to involve the condensation of a thiazole precursor with the pyrimidine to give an intermediate which is then converted to thiamin. According to our biosynthetic scheme, compound I (Figure 5) would represent this thiazole precursor and compound III (Figure 5) would represent the condensed intermediate, which is subsequently converted to thiamin.

The second *Neurospora* pathway was postulated to involve the direct condensation of the preformed thiazole II with the pyrimidine. This second pathway is, therefore, analogous to the established pathway for thiamin biosynthesis in yeasts (Lewin and Brown, 1961). The major requirement of this pathway is that the nonsubstituted thiazole II holds an intermediate position in the pathway. This thiazole could possibly be produced by the metabolism of the tyrosine to the C-2 aldoxime which, in turn, could incorporate sulfur to form the thiohydroximic acid. The resulting N-C-S unit could then, either directly or indirectly after rearrangement to the thiocyanate, supply the C-2 and the nitrogen and sulfur atoms of the thiazole. Although these reactions have not been described for microbial systems, they have been studied extensively in higher plants in relation to the biosynthesis and reaction of glucosinolates and may shed some light on the final resolution of thiazole biosynthesis in nature (Mahadevan, 1973).

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