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DETERMINATION OF THE METABOLIC ORIGIN OF THE SULFUR ATOM IN THIAMIN OF ESCHERICHIA COLI BY MASS SPECTROMETRY

Edward DeMoll[†] and William Shive

Clayton Foundation Biochemical Institute and
Department of Chemistry, University of Texas at Austin
Austin, Texas 78712-1096

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In this study cells were grown in ^{34}S-sulfate or L-[sulfane-^{34}S]thiocystine, and the effects of unlabeled methionine and cystine on incorporation of sulfur into methionine, cystine and thiamin were determined. Unlabeled methionine effectively suppresses the incorporation of ^{34}S into methionine but not into cysteine or thiamin. In contrast, cystine blocks incorporation of ^{34}S only to approximately the relative ratio of ^{32}S to ^{34}S indicating, that cysteine is closely related to the origin of the sulfur in thiamin, and therefore the sulfane sulfur of thiocystine is also an effective source of the thiamin sulfur.

While progress has been made in elucidating the pathway for the biosynthesis in bacteria of the thiazole moiety of thiamin, much still remains in doubt. Previous investigations have shown that carbon-2 of the thiazole ring is derived from C-2 of tyrosine in Escherichia coli (1) and in Salmonella typhimurium (2). White and Rudolph (3) demonstrated that tyrosine is also the source of the nitrogen atom. White (4) later showed that tyrosine is cleaved with elimination of p-hydroxybenzyl alcohol, most likely through an unstable quinone methide intermediate. From stable isotope incorporation studies White (5,6) was able to propose that a pentulose derivative might be the precursor for the other five carbon atoms of the thiazole

[†]Present address: Laboratory of Biochemistry, National Heart, Lung, and Blood Inst., Bethesda, MD 20205.

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moiety. This has been supported by David et al. (7) who were able to incorporate deuterated 1-deoxy-D-threo-pentulose into the thiazole portion of thiamin of E. coli.

The source of the sulfur atom has been studied in both E. coli (8) and S. typhimurium (9). Estramareix et al. (8) found that addition of 1mM glutathione to cultures containing 0.5 mM $^{35}SO_4^{-2}$ caused an approximately 50% lowering of thiamin specific activity, while neither methionine nor homocysteine had any significant (<6%) effect, thereby implicating cysteine as the possible sulfur donor. Bellion and Kirkley (9) added various sulfur-containing compounds to cultures with ^{35}S-cysteine. They found that glutathione caused a 12% increase, homocysteine a 15% decrease, and methionine a 46% decrease in 4-methyl-5-β-hydroxyethyl thiazole specific activity, but concluded, however, that these differences were not significant, and that these compounds do not compete with cysteine in providing the sulfur atom in thiamin biosynthesis. Neither of these investigations excluded the possibility of the sulfur atom of thiamin originating with sulfide.

EXPERIMENTAL PROCEDURES

Materials. All amino acids, thiamin and trifluoroacetic anhydride were purchased from Sigma. ^{34}S at 90 atom % was obtained in elemental form from Monsanto (^{32}S, 5.44 atom %; ^{33}S, 2.02 atom %; ^{34}S, 90.83 atom %; ^{36}S, 1.71 atom %) and ^{34}S-sodium sulfate from Prochem, U.S. Services, Inc. (^{32}S, 3.27 atom %; ^{33}S, 2.66 atom %; ^{34}S, 93.16 atom %; ^{36}S, 0.92 atom %). E. coli K-12 hpbλ$^-$ was a kind gift from Dr. C. H. Pai of the University of Calgary, Calgary, Alberta, Canada. L-[sulfane-^{34}S]Thiocystine[1] was synthesized as previously reported (10) from cysteine and elemental sulfur.

Growth of the organism. E. coli was grown on an inorganic salts-glucose medium enriched with 16 amino acids as described elsewhere (10). The medium was supplemented with 300-360 μM sulfur in the form of SO_4^{-2}, L-methionine, L-cystine, or L-thiocystine as indicated. Due to contaminating S^{-2} or SO_4^{-2} in the chemicals comprising the growth medium, presumably with isotopic abundances of naturally occurring sulfur, the isotopic distributions were changed slightly from

[1] bis(2-amino-2-carboxyethyl)trisulfide.

those listed above. For example, the theoretical isotopic abundances for the medium containing only 90 atom % $^{34}SO_4$ were calculated (from S^{-2} or SO_4^{-2} assay values of the reagents used in the medium) to be as follows: ^{32}S, 6.69 atom %; ^{33}S, 2.59 atom %; ^{34}S, 89.85 atom %; ^{36}S, 0.89 atom %.

The media were sterilized by autoclaving at 18 lb/in^2 pressure for 5 min, cooled, and then seeded with approximately 1 mg of cells from an inoculum grown overnight in the basal medium supplemented with unlabeled cystine and methionine. The culture was then incubated at 37°C at a rotary speed of 180 rpm for about 24 h in a New Brunswick controlled environment incubator shaker.

Isolation and Derivatization of Amino Acids and Thiamin. Cells were harvested by centrifugation at 6000 g and washed twice with 0.9% NaCl. The cells were resuspended in 8 ml of 0.1 N HCl and then placed in a boiling water bath for 20 min. Following centrifugation the pellet was used for amino acid determination. The supernatant was cooled to room temperature and 1 ml of 1 M sodium acetate buffer, pH 4.7, was added. The pH was then adjusted to 4.7 with 2 M KOH. Dephosphorylation, purification, and derivatization then proceded as described previously (3). Amino acid purification and derivatization was performed according to the procedures of DeMoll et al. (11).

Gas Chromatography-Mass Spectrometry. This work was performed on a Finnegan 4023 gas chromatograph-mass spectrometer with an INCOS data system. The thiamin derivative, 4-methyl-5-β-hydroxyethylthiazole, was chromatographed isothermally at 125°C on a 50 m SE fused sililca capillary column. Elution of the thiamin derivative was followed by scanning for ions of m/z 125. A known sample of thiamin, treated as described above, produced a peak with the same retention time and mass spectrum as the unknown. Mass spectra of ^{34}S-enriched and unenriched 4-methyl-5-β-hydroxyethylthiazole are shown in Figure 1. Amino acids were

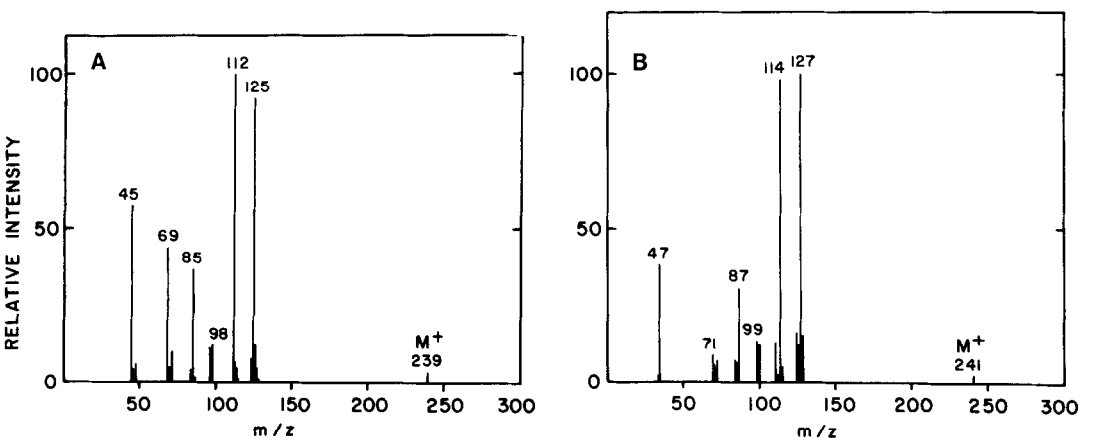

Figure 1. Mass spectra of 4-methyl-5-β-hydroxyethylthiazole isolated from E. coli grown in media of natural abundance ^{34}S (A) and 90 atom percent ^{34}S sulfate (B) as described in Experimental Procedures.

isolated and identified as described previously (11). All spectra were recorded at 70 eV with an injection temperature of 250°C and an ion source of 270°C.

Measurement of Isotope Distribution. It has been shown previously that there is a lack of amino acid compartmentalization and protein turnover in growing bacterial cells (12,13). Consequently the isotope distribution of ^{32}S, ^{33}S, ^{34}S, and ^{36}S from cellular protein should reflect the average isotopic distribution of these sulfur isotopes throughout the growth of the organism.

Experimental isotopic abundances of sulfur were obtained from the m/z 61 ($CH_2SCH_3^+$) ion cluster for the cysteine and methionine derivatives (11,14). The m/z 125 ion cluster was chosen for calculation of the isotopic abundance of sulfur in thiamin as this cluster contained sulfur, was of high relative intensity, and was more reproducible than the m/z 112 cluster. The origin of these ions has been indicated previously (3). Atom percent ^{34}S was calculated with the aid of a microcomputer and three computer programs that could construct hypothetical mass spectra for the m/z 61, 112, and 125 ion clusters (11) discussed above.

RESULTS AND DISCUSSION

The data in Table 1 show that methionine can be eliminated from consideration as a source of the sulfur atom of thiamin. Additionally, it may be seen that the incorporation of $^{34}SO_4^{-2}$ into cysteine, thiamin, and methionine is lowered a similar amount in each case by exogenous cystine. While these data support the idea of cysteine being the sulfur source for thiamin, they do not rule out sulfide as a possibility. The experiments illustrated in Table 2 indicate

Table I: Incorporation of ^{34}S from $^{34}SO_4^{-2}$ into Thiamin, Cysteine, and Methionine[a]

Expt.	atom % excess ^{34}S in Thiamin	Cysteine	Methionine
1	84.7±0.9	85.9±0.8	87.1±0.5
2	86.9±1.2	85.8±0.5	5.9 ±1.3
3	51.8±1.2	50.2±0.6	44.7±1.0

[a]The organism was grown in the presence of the indicated sulfur sources: Expt. 1, 310 μM $^{34}SO_4^{-2}$; Expt. 2, 155 μM $^{34}SO_4^{-2}$, 155 μM L-methionine; Expt. 3, 155 μM $^{34}SO_4^{-2}$, 77.5 μM L-cystine. Values for atom percent excess ^{34}S were obtained as described in Experimental Procedures. Data are expressed as mean ± standard deviation.

Table II: Incorporation of ^{34}S from L-[sulfane-^{34}S]thiocystine into Thiamin, Cysteine, and Methionine [a]

Expt.	atom % excess ^{34}S in		
	Thiamin	Cysteine	Methionine
1	22.6±1.3	23.5±0.6	2.0±0.9
2	14.6±0.8	16.4±1.1	<2
3	10.5±1.0	10.5±0.5	<2
4	<2	<2	<2

[a]The organism was grown in the presence of the indicated sulfur sources: Expt. 1, 50μM L-[sulfane-^{34}S]thiocystine, 150 μM L-methionine; Expt. 2, 40 μM L-[sulfane-^{34}S]thiocystine, 30μM L-cystine, 180 μM L-methionine; Expt. 3, 20 μM L-[sulfane-^{34}S]-thiocystine, 60 μM L-cystine, 180 μM L-methionine; Expt. 4, 2 μM L-[sulfane -^{34}S]thiocystine, 87 μM L-cystine, 180 μM L-methionine.

that the sulfane sulfur of thiocystine is incorporated to about equal extents into cysteine and thiamin, and cystine reduces the incorporation in proportion to the combined unlabeled sulfur in cystine and thiocystine. White (15) has reported that L-[sulfane-^{34}S]thiocystine labels cysteine and sulfide differentially with ^{34}S abundance being higher (approximately 50 atom %) in sulfide. Therefore the lack of such differential labeling in these experiments tends to eliminate sulfide as a direct precursor of thiamin.

It is apparent then, from this and previous studies, that in *E. coli* the biosynthesis of sulfur-containing compounds proceeds via cysteine or a closely related intermediate in every case so far studied. This has been shown to be true for lipoic acid (16), biotin (10,11), iron-sulfur centers (17), coenzyme A (18), methionine (19) and now thiamin.

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