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THE ORIGIN OF SULFUR IN BIOTIN

Edward DeMoll and William Shive

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

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The comparative ability of *Escherichia coli* K-12 hpb λ^-, a biotin overproducing strain, to incorporate ^{35}S from isotopically labeled L-methionine, L-cystine, and the sulfane sulfur of thiocystine was determined. Comparison of the specific activity of sulfur in biotin produced by the organism with that of the ^{35}S-labeled amino acids demonstrates that the sulfur of cystine is transferred to biotin with an efficiency of at least 75%, that the sulfur of methionine does not contribute to biotin significantly, and that the sulfane sulfur of thiocystine contributes approximately one-third of the sulfur to newly synthesized biotin and is essentially equivalent in utilization to that of the other two sulfur atoms in the molecule.

The final step in the biosynthesis of biotin has been shown to involve the conversion of dethiobiotin into biotin (1); however, the source of the sulfur atom remains in doubt. Investigators working with *Saccharomyces cerevisiae* (2) and *Rhodotorula glutinis* (3) have reported the incorporation of sulfur from [^{35}S]methionine into biotin, but since they failed to determine the specific activities of the [^{35}S]biotin, their results are not conclusive.

Recently Frappier and Marquet tested the ability of L-[^{35}S]cysteine to provide the sulfur atom for biotin biosynthesis in Achromobacter IVSW but observed no significant incorporation of ^{35}S (4).

As a part of our investigations concerning sulfur metabolism in *Escherichia coli*, a study of the contribution of sulfur from cystine, methionine, and thiocystine[1] was undertaken.

MATERIALS AND METHODS

Sources of materials obtained commercially are as follows: L-[^{35}S]cystine and L-[^{35}S]methionine from New England Nuclear, L-[^{35}S]cysteine

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

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hydrochloride from Amersham, all non-radioactive amino acids, avidin, and D-biotin from Sigma, and Biotin Assay Medium from Difco.

L-[sulfane-^{35}S]Thiocystine was synthesized as described by Fletcher and Robson (5) from cysteine and elemental sulfur. A copy of *E. coli* K-12 hpb λ^- was a kind gift from Dr. C. H. Pai of The University of Calgary, Calgary, Alberta. Dialysis tubing was prepared as described previously (6). Scintillation fluid was formulated according to the method of Pande (7).

Growth of the organism. The culture medium was an inorganic salts-glucose mixture supplemented with 16 amino acids and consisted of 10 g of glucose, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1.35 g of $MgCl_2 \cdot 6\ H_2O$, 1 g of NH_4Cl, 0.5 g of NaCl, 1.5 mg of $Fe(NH_4)_2(SO)_4 \cdot 6\ H_2O$, and 25 mg each of L-alanine, L-arginine hydrochloride, L-aspartic acid, L-glutamic acid, glycine, L-histidine hydrochloride, L-isoleucine, L-leucine, L-lysine hydrochloride, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine per liter of solution. The medium was sterilized by autoclaving for 5 min at 121°C and 18 lb/in^2. Solutions of L-cystine, L-cysteine, L-thiocystine, and L-methionine were sterilized by passage through a 0.2 micron nitrocellulose filter and were added to the culture medium as indicated. The medium (1 l) was inoculated with approximately 1 mg of cells and incubation was carried out at 37°C in a New Brunswick Controlled Environment Incubator Shaker at a rotary speed of 180 rpm for about 24 hours. By this time, the level of biotin excreted into the medium had reached its maximum.

Isolation of biotin. *E. coli* cells were sedimented by centrifugation at 6000 x g for 5 min. The supernatant fraction, containing biotin, was decanted and the cells were discarded. Saturated lead acetate solution (35 ml per liter of supernatant) was added and the white precipitate removed by centrifugation at 6000 x g for 5 min. The liquid was acidified with H_2SO_4 (6 ml per liter) to remove excess lead, and the pH of this solution was then adjusted to 3.0 with solid KOH. One gram of activated charcoal was added to each liter of this solution, and the suspension was stirred 4 h at room temperature to adsorb the biotin. The charcoal was then removed on Whatman No. 2 filter paper by vacuum filtration and washed with 150 ml of water. Biotin was eluted from the charcoal by stirring with 160 ml of a solution composed of eighteen volumes of 50% ethanol and one of concentrated NH_4OH. After 4 h the filtrate was separated from the charcoal by vacuum filtration through Whatman No. 2 paper. The filtrate was saved and the charcoal stirred once more as before with the ethanol-ammonia mixture. The solution was filtered again, and the two filtrates were combined. The charcoal eluate was concentrated to a syrup *in vacuo* at 40°C, then extracted with 90 ml of absolute ethanol. The precipitate formed was removed partially by centrifugation in a clinical centrifuge, then the remainder was removed by filtration through a 0.2 micron nitrocellulose filter. The ethanol was completely evaporated *in vacuo* at 40°C, and the residue was dissolved in 6 ml of 0.2 M $(NH_4)_2CO_3$. Any undissolved residue was removed by filtration through a 0.2 micron nitrocellulose filter.

Determination of biotin specific activity. A portion of the crude biotin preparation was reserved and tested for biotin content with an assay employing *Lactobacillus plantarum* as described by Wright and Skeggs (8). The remainder

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[2] unpublished results

of the crude biotin preparation was divided into two equal parts. To the first was added 20 units of avidin per liter of original supernatant fraction and 20 mg biotin in 1 ml of 0.2 M $(NH_4)_2CO_3$. To the second portion an identical amount of avidin was added, but the unlabeled biotin was omitted, and 1 ml of 0.2 M $(NH_4)_2CO_3$ included instead. Each portion was dialyzed separately for a total of 36 h against two changes of 4 l of 0.2 M $(NH_4)_2CO_3$ at room temperature. At the end of this period, the contents of the dialysis bags were removed and their volumes recorded. Each sample, typically 4 ml, was mixed with 15 ml of scintillation fluid, shaken, and finally counted in a Beckman LS-100 scintillation counter. Allowances were made to correct for possible effects of quenching by bringing all samples to the same volume with 0.2 M $(NH_4)_2CO_3$. By combining the values obtained from the biotin assay and the liquid scintillation counting (see Results), a specific activity with units of cpm/mole was calculated. The specific activity of biotin was then compared to that of the particular ^{35}S-labeled compound being studied and a value for percent incorporation of the ^{35}S into biotin was obtained.

RESULTS

These experiments follow the incorporation of ^{35}S from L-[^{35}S]-methionine, L-[^{35}S]cystine, and L-[sulfane-^{35}S]thiocystine into biotin. In the first instance *E. coli* was grown in the presence of L-[^{35}S]methionine and unlabeled cystine. The data are shown in Table 1, Experiment 1. Essentially none of the sulfur from methionine was incorporated into biotin. However, when the label was in cystine and not in methionine (Table 1, Experiments 2 and 3), approximately 80% of biotin was calculated to be labeled with ^{35}S. L-Thiocystine may be used as a sulfur source for *E. coli*, and all of the sulfur atoms are utilized by the organism[2]. When *E. coli* was grown in the presence of L-[sulfane-^{35}S]thiocystine, an average of 28.7% of the label was found to be transferred to biotin (Table 1, Experiment 4). This value is approximately 86% of the value expected if all sulfur atoms are equivalent in their contributions toward biotin biosynthesis.

Since cystine and thiocystine appeared to be equally effective in serving as a sulfur source for biotin biosynthesis, further experiments, similar to those illustrated in Table 1, were performed. When *E. coli* was grown in the presence of 50 μM L-thiocystine and 75 μM L-[^{35}S]cystine, approximately 44% of the biotin was determined to have incorporated ^{35}S. This compares with the theoretically possible 50% if all sulfur was utilized equally. Additionally, when the label originated in the sulfane sulfur of thiocystine and

TABLE 1

INCORPORATION OF THE SULFUR FROM METHIONINE, CYSTINE, AND THIOCYSTINE INTO BIOTIN

	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Total Biotin in Sample (nmole)[a]	66.8	61.0	74.9	46.6
^{35}S in Portion Containing 1000X Unlabeled Biotin (cpm)	1.03×10^3	3.52×10^2	2.56×10^3	5.35×10^2
^{35}S in Portion Containing no Unlabeled Biotin (cpm)	1.16×10^3	2.13×10^3	4.05×10^4	5.41×10^3
^{35}S in Biotin (cpm)	1.3×10^2	1.78×10^3	3.79×10^4	4.88×10^3
Specific Activity of Biotin (cpm/mole)	1.946×10^9	2.92×10^{10}	5.07×10^{11}	1.047×10^{11}
Specific Activity of ^{35}S-labeled Precursor (cpm/mole)	3.04×10^{11}	3.73×10^{10}	6.24×10^{11}	3.65×10^{11}
% Incorporation into Biotin	0.64	78.3	81.2	28.7[b]

[a] From assay with *L. plantarum*

[b] Based upon the sulfane sulfur only

In each experiment the results presented are the averages of data from three separate assays. In each case *E. coli* cells were grown under the conditions described in Materials and Methods. Experiment 1: 150 μM L-[^{35}S]methionine and 75 μM L-cystine. Experiments 2 and 3: 75 μM L-[^{35}S]cystine and 150 μM L-methionine. Experiment 4: 100 μM L-[sulfane-^{35}S]thiocystine.

unlabeled cystine was added, the ^{35}S label was diluted out of biotin on a basis proportional to the total sulfur present in cystine and thiocystine. That the theoretical maximum of 100% incorporation was not reached in any of the experiments, we interpret as being due to technical problems inherent in our procedure. We attribute this, at least in part, to the loss of a portion of the [^{35}S]biotin, which does occur during dialysis and transfer to scintillation vials of the solution containing the [^{35}S]biotin. Since the biotin assay is performed on the crude biotin preparation prior to dialysis, any loss after this point would reduce the calculated specific activity of the biotin.

The possibility also exists that since the crude biotin preparation contains a mixture of nutrients, *L. plantarum*, the biotin assay organism, might be responding to something other than biotin. This would raise the apparent biotin level, and therefore lower the calculated specific activity of the [^{35}S]biotin.

Attempts to alleviate this source of error by performing the biotin assay subsequent to dialysis, rather than prior to it, were limited by the difficulty of recovery of all of the biotin from the avidin-biotin complex.

Experiments performed to test the ability of *E. coli* to incorporate ^{35}S from L-[^{35}S]cysteine were consistent with those reported earlier (9). When the cells were grown with 1.5 mM sulfate and 30 μM L-[^{35}S]cysteine, we saw less than 10% incorporation of ^{35}S into biotin if the cells were harvested early in exponential growth. Without sulfate growth was poor initially, but if the cells were allowed time to grow, the incorporation of ^{35}S into biotin rose to approximately 65%.

DISCUSSION

Attempts to discover the source of the sulfur atom in biotin (1,2) have been limited by the failure of the investigators to measure the specific activity of the newly synthesized biotin and then compare it to that of the precursor compound in question. In these previous studies small amounts of ^{35}S were found to be incorporated into biotin when [^{35}S]methionine provided the radioactive label. If *Saccharomyces cerevisiae* and *Rhodotorula glutinis* metabolize sulfur in a manner similar to *E. coli*, then the small amounts of incorporation obtained might be accounted for by sulfur from degraded methionine, which had been recycled into cysteine.

Frappier and Marquet (4) reported that they were unable to incorporate the label from L-[^{35}S]cysteine into biotin synthesized by Achromobacter IVSW. However, they were able to demonstrate that biotin is synthesized by this organism from dethiobiotin, according to the pathway found in other microorganisms (1). The inability of Achromobacter to incorporate ^{35}S from cysteine might be due to the failure of the organism to use exogenous cysteine

as a sulfur source altogether as is true for *E. coli*. (9). Roberts, *et al*. have shown that exogenously supplied cysteine must first be oxidized to cystine before it will be transported and utilized by *E. coli* We have confirmed these results. In fact, we were able to block almost all incorporation of ^{35}S into biotin from L-[^{35}S]cysteine by growing the cells in a medium containing 1.5 mM sulfate and 30 μM L-[^{35}S]cysteine. When sulfate was left out the cells grew poorly at first, but after a lag of several hours (presumably due to the slow formation of cystine) the cells appeared to grow normally and were able to incorporate the ^{35}S label into biotin. Micromolar amounts of cysteine apparently cannot be oxidized quickly enough under standard conditions to provide an adequate supply of sulfur to rapidly growing *E. coli*, so when millimolar amounts of sulfate are also present, the organism will use sulfate. When given an adequate supply of cystine, *E. coli* will use it to the near exclusion of exogenous sulfate, sulfite, or sulfide (9). Frappier and Marquet (4) employed a medium which contained 8 mM sulfate and 12 μM L-[^{35}S]cysteine, so if Achromobacter metabolizes sulfur as *E. coli* does, then the above explanation could account for their inability to see incorporation of ^{35}S from cysteine into biotin.

Our results clearly show that in *E. coli*, the sulfur atom in biotin is derived from exogenously supplied cystine. Thiocystine is also able to supply this sulfur atom equally well from either of its two kinds of sulfur atoms. Whether or not cysteine is the actual sulfur donor in the conversion of dethiobiotin to biotin is debatable, but cysteine can surely serve as a relatively direct precursor of the sulfur atom. In order to confirm the results presented here, we are also investigating this final step in biotin biosynthesis by mass spectrometric methods. In these studies comparison of ^{34}S incorporation from L-[sulfane-^{34}S]thiocystine into internal cysteine, methionine, and excreted biotin provide results which are in agreement with the data presented above. We have supplied a sample of L-[sulfane-^{34}S]-thiocystine to Robert H. White of Virginia Polytechnic Institute. By allowing *E. coli* to use this compound as its sole sulfur source, he has obtained data

which are interpreted to indicate that all of the sulfur atoms of thiocystine are cycled through cysteine (10).

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