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Determination of the Metabolic Origins of the Sulfur and 3′-Nitrogen Atoms in Biotin of *Escherichia coli* by Mass Spectrometry†

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ABSTRACT: Two steps in the biosynthesis of biotin in *Escherichia coli*, incorporation of the nitrogen atom of methionine into 7-keto-8-aminopelargonic acid and of the sulfur atom into dethiobiotin, were examined. Sulfur and nitrogen metabolism were monitored by gas chromatography–mass spectrometry of volatile derivatives of internal (protein-bound) amino acids and excreted biotin. We were able to show that internal cysteine and excreted biotin were labeled to the same extent with ^{34}S from either of two exogenous sulfur sources, $^{34}SO_4^{2-}$ or L-[*sulfane*-^{34}S]thiocystine. Internal methionine was eliminated from consideration, while cysteine, or possibly a closely related intermediate, was implicated as providing the sulfur atom for biotin biosynthesis. Also, in experiments designed to follow the metabolism of the nitrogen atom of methionine, it was found that biotin excreted into the culture medium by this organism grown with 95 atom % [^{15}N]methionine contained greater than 70 atom % excess ^{15}N in one of the nitrogens over that obtained from cultures grown with methionine of natural abundance ^{15}N. These results provide evidence for the direct transfer of the methionine nitrogen as the role of *S*-adenosylmethionine in the conversion of 7-keto-8-aminopelargonic acid to 7,8-diaminopelargonic acid.

As shown previously (DeMoll & Shive, 1982, 1983), *Escherichia coli* will employ exogenous cystine, to the exclusion of methionine, as a sulfur source for biotin biosynthesis. Also, it was determined that all of the sulfur atoms of thiocystine [bis(2-amino-2-carboxyethyl) trisulfide] contribute approximately equally toward biotin biosynthesis. White (1982) has shown that both types of the sulfur atoms of thiocystine are eventually cycled through cysteine when the former is used as the sole sulfur source for the growth of the organism. Evidence has been presented that once transported into the organism, cystine is metabolized by both direct reduction to cysteine and by β-elimination to yield thiocysteine, pyruvate, and ammonia (White, 1983). Consequently, it was necessary to extend our investigation to the internal cysteine pool of *E. coli* to ensure that the ^{35}S incorporation into biotin we had previously observed was due to reduction of cystine to cysteine followed by (perhaps several steps later) transfer of sulfur to biotin.

Methionine has been shown by Pai (1971) to be 7–20 times as effective as any other amino acid in the production of 7,8-diaminopelargonic acid from 7-keto-8-aminopelargonic acid by crude extracts from several strains of *E. coli* K-12. A requirement for pyridoxal phosphate or pyridoxamine or its phosphate indicated the possibility of transamination. Eisenberg & Stoner (1971) have since shown that the ability of methionine to stimulate the in vitro synthesis of 7,8-diaminopelargonic acid is dependent upon ATP and Mg^{2+} and that *S*-adenosylmethionine is about 10 times as effective as methionine, ATP, and Mg^{2+}. In a purified system, methionine, ATP, and Mg^{2+} were shown to be ineffective in replacing *S*-adenosylmethionine. The direct participation of *S*-adenosylmethionine in a transamination reaction was proposed. However, attempts to detect the keto acid derived of *S*-adenosylmethionine were unsuccessful, and the possibility that *S*-adenosylmethionine might have an essential role in the reaction without transfer of nitrogen must be considered.

In this investigation, procedures for determining the atom percent excess ^{34}S and ^{15}N in excreted biotin, using gas chromatography–mass spectrometry techniques, were devel-

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oped. Biotin and cysteine were labeled to the same extent with ^{34}S from sulfate or from the sulfane sulfur of thiocystine under a variety of conditions, providing further evidence for the close relationship of cysteine to the biogenesis of biotin. Also, [^{15}N]methionine was found to contribute the major part of the 3′-nitrogen of biotin synthesized by *E. coli*.

Experimental Procedures

Materials. All amino acids and *N,O*-bis(trimethylsilyl)-acetamide 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 90 atom % $Na_2{}^{34}SO_4$ 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 %). DL-[^{15}N]Methionine (95 atom % ^{15}N) was purchased from Merck and Co. *E. coli* K-12 hpb λ^-, a biotin-overproducing strain, was a kind gift from Dr. C. H. Pai of The University of Calgary, Calgary, Alberta, Canada.

Growth of the Organism. E. coli K-12 hpb λ^- was grown in an inorganic salts–glucose medium enriched with 16 amino acids as described elsewhere (DeMoll & Shive, 1983). 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 the presence of contaminating HS^- 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 that listed above. Theoretical isotopic abundances for the ^{34}S-enriched SO_4^{2-} were calculated from S^{2-} or SO_4^{2-} assay values of the reagents used in the medium and were found to be as follows: ^{32}S, 6.69%; ^{33}S, 2.59%; ^{34}S, 89.85%; ^{36}S, 0.89%. For determination of the extent of incorporation of the nitrogen of methionine into biotin, this basal medium was supplemented with cystine and DL-[^{15}N]methionine to provide final concentrations of 87.5 and 175 μM, respectively.

The medium (1 L) was sterilized by autoclaving at 18 lb/in.^{2} pressure for 5 min, cooled, and then seeded with approximately 1 mg of cells of *E. coli* K-12 hpb λ^- 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. By this time, the level of biotin excreted into the medium had reached its maximum.

Isolation and Derivatization of Amino Acids and Biotin. Cells were harvested by centrifugation at 6000*g*, and the supernatant fraction was reserved for isolation of biotin. The cell pellet was used for amino acid isolation, which was performed according to the procedures described by White & Rudolph (1978) and White (1981).

Biotin was concentrated by adsorption of the excreted biotin on activated charcoal. This was followed by elution of biotin from the charcoal with an ethanol–ammonia solution as described previously (DeMoll & Shive, 1983). This charcoal eluate was concentrated to a syrup in vacuo at 40 °C; then, it was extracted with 90 mL of 75% ethanol. The ethanol solution was filtered through a 0.2-μm nitrocellulose filter and concentrated to a volume of approximately 10 mL. This solution was applied to an 8.5 × 2.6 cm column of Dowex AG 50W-X8, 200–400 mesh (H^+ form). Fractions of 20 mL each, collected at a flow rate of 12 mL/min, were eluted with water. Fractions 11–28, which contained biotin, were pooled and concentrated. The crude biotin preparation was evaporated to dryness in a small vial with a stream of air at 40 °C. The tris(trimethylsilyl) derivative of biotin was then prepared by introducing 20 μL of pyridine and 20 μL of *N,O*-bis(trimethylsilyl)acetamide into the vial and then heating the capped vial for 15 min at 95 °C.

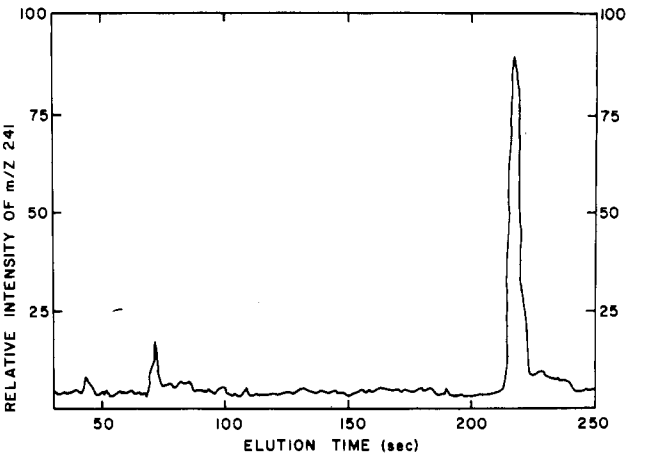

FIGURE 1: Separation of the tris(trimethylsilyl) derivative of biotin by gas chromatography. Under the conditions employed, which are described in the text, the biotin derivative eluted in approximately 215 s.

Gas Chromatography–Mass Spectrometry. This work was performed on a Finnigan 4023 gas chromatograph–mass spectrometer with an INCOS data system. The tris(trimethysilyl) derivative of biotin was chromatographed isothermally at 235 °C on a 6 ft × 2 mm glass column packed with 3% SP2100. The gas chromatographic trace seen in Figure 1 shows that although the biotin was not entirely pure, its elution from the column was readily identifiable by scanning for ions of *m/z* 241. This is the ion of greatest intensity and is of sufficient size and complexity to allow positive identification of the biotin derivative. A known sample of biotin produced a peak with the same retention time and mass spectrum as the unknown.

The *N*-(trifluoroacetyl) *n*-butyl esters of the amino acids were separated on a 50-m SE fused silica capillary column by employing a temperature program from 130 °C at 5 °C/min. 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. The proportion of $^{34}S/^{32}S$ in methionine and cysteine and of $^{15}N/^{14}N$ in methionine from cellular protein should represent the average isotopic distribution of sulfur and nitrogen in these amino acids during the growth period of the organism. This is reasonable in light of previous work (Rotman & Spiegelman, 1954; Koch & Levy, 1955) that showed the lack of amino acid compartmentalization and protein turnover in growing bacterial cells. The $^{34}S/^{32}S$ and $^{15}N/^{14}N$ ratios in biotin collected in the supernatant fraction should also reflect the average isotopic abundances of sulfur and nitrogen during the growth of the cells, as biotin is excreted continuously at a rate directly proportional to cell growth (results not shown).

Experimental isotopic abundances of sulfur were obtained from the *m/z* 61 ($CH_2SCH_3^+$) ion cluster for the cysteine and methionine derivatives (White, 1981). The *m/z* 445 ion cluster was chosen for calculation of the isotopic abundance of sulfur in biotin, because the only other observed sulfur-containing ion cluster was that of the molecular ion *m/z* 460, which was of such low intensity as to make it useless for any of the determinations.

Atom percent excess ^{34}S was calculated with the aid of a microcomputer and two computer programs that could construct hypothetical spectra for the *m/z* 61 and 445 ion clusters. The relative intensities of the ions in these spectra were based upon varying, known proportions of ^{32}S, ^{33}S, ^{34}S, and ^{36}S.

Construction of hypothetical spectra was continued, with a change in sulfur isotope proportions each time until the best match to the experimentally derived spectrum was obtained. The best match was chosen by comparing the two sets of intensities in the following manner:

$$R = 1 - [[(I^c_M - I^x_M)^2 + (I^c_{M+1} - I^x_{M+1})^2 + \ldots + (I^c_{M+n} - I^x_{M+n})^2]^{1/2}]/[(n+1) \times 100]$$

where I^c and I^x are the relative intensities of the calculated and experimentally derived values, respectively, for the various ions of the isotopic cluster consisting of M, M + 1, ..., and M + n. R is the correlation coefficient between the experimentally derived and computer-generated values being examined and, for the best match, typically was greater than 0.990. This procedure, which will be discussed at greater length in a later publication, was chosen for sulfur isotope determinations instead of those described previously (Campbell, 1974; White, 1981) because of its greater accuracy. Calculation of atom percent excess ^{34}S by either of these methods produced values 3–6% higher than those obtained by the procedure described above.

Atom percent excess ^{15}N was calculated upon the basis of the intensities of the ions of m/z 241 and 242 for the biotin derivative and m/z 171 and 172 for the methionine derivative. The ion cluster around the m/z 241 fragment was chosen for the calculation of atom percent excess ^{15}N in biotin, because it offered the highest relative intensities and reproducibility of any of the nitrogen-containing ion clusters (m/z 460, 445, 241, 204, and 189). The choice was made of the ion cluster m/z 171 of the methionine derivative for similar reasons. In the cases of the other amino acids, ion clusters were chosen that had high relative intensities yet still retained nitrogen.

Results and Discussion

Sulfur Incorporation. It has been shown that *E. coli* requires a minimum of approximately 300–400 μM sulfur in a usable form for optimal growth (Roberts et al., 1955). To ensure that the organism could not exercise selection of one form of sulfur over another, *E. coli* was grown in the presence of a growth-limiting amount of usable sulfur in each experiment. In this way, the organism would be forced to mobilize all available forms of sulfur present in the medium. Roberts et al. (1955) also reported that the sulfur derived from a variety of sources including SO_4^{2-}, SO_3^{2-}, HS^-, L-cystine, DL-lanthionine, *S*-methylcysteine, or glutathionine provided maximum potential growth. We reported that L-thiocystine can be a good sulfur source for *E. coli* (DeMoll & Shive, 1983), and White (1982) has presented evidence that approximately half of each type of the sulfur atoms of L-thiocystine are cycled through de novo synthesized cysteine. While *E. coli* may be able to use a variety of exogenous sulfur sources, the internal sulfur reservoirs in the organism have been shown to consist almost entirely of cysteine and methionine in protein and glutathione (Roberts et al., 1955). The remaining sulfur resides in compounds such as coenzyme A, lipoic acid, thiamin, and biotin.

In this investigation, possible roles for internal sulfur pools of cysteine and methionine in contributing the sulfur atom in biotin biosynthesis were examined. The results of this study are summarized in Table I. In all applicable experiments, cystine, rather than cysteine, was used as a sulfur source. Cysteine has been shown to inhibit the growth of *E. coli* and to be transported only subsequent to its oxidation to cystine (Roberts et al., 1955; DeMoll & Shive, 1983). It is readily apparent from the results in Table I that while exogenous methionine was capable of lowering $^{34}SO_4^{2-}$ incorporation into cellular methionine, it did not affect the ^{34}S level in biotin or cysteine (experiment 2). Incorporation of $^{34}SO_4^{2-}$ into biotin was reduced only by inclusion of exogenous cystine (experiment 3), which also lowered ^{34}S incorporation into internal cysteine by a similar amount. Mass spectra of the enriched and unenriched cysteine and biotin derivatives are shown in Figures 2 and 3. Additionally, these results also demonstrate the absence of significant reversibility of the reactions that convert cysteine into methionine in *E. coli*. This has been shown previously by Delavier-Klutchko & Flavin (1965).

We also investigated a possible role for the sulfane sulfur of thiocystine in biotin biosynthesis. The results of these experiments are summarized in Table II. The sulfur atom of cystine and both types of sulfur atoms of thiocystine are rapidly assimilated, apparently without distinct preference, into cysteine and biotin synthesized by the cells. When the ex-

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

expt no.	sulfur source	atom percent excess ^{34}S in biotin	cysteine	methionine
1	310 μM $^{34}SO_4^{2-}$	87.7 ± 0.4	85.9 ± 0.8	87.1 ± 0.5
2	155 μM $^{34}SO_4^{2-}$, 155 μM L-methionine	85.6 ± 0.9	85.8 ± 0.5	5.9 ± 1.3
3	155 μM $^{34}SO_4^{2-}$, 77.5 μM L-cystine	48.0 ± 1.3	50.2 ± 0.6	44.7 ± 1.0

[a] *E. coli* hpb λ⁻ was grown in the presence of the indicated sulfur sources, and values for atom percent excess ^{34}S were obtained as described under Experimental Procedures. Data are expressed as mean ± standard deviation, n = 3–5.

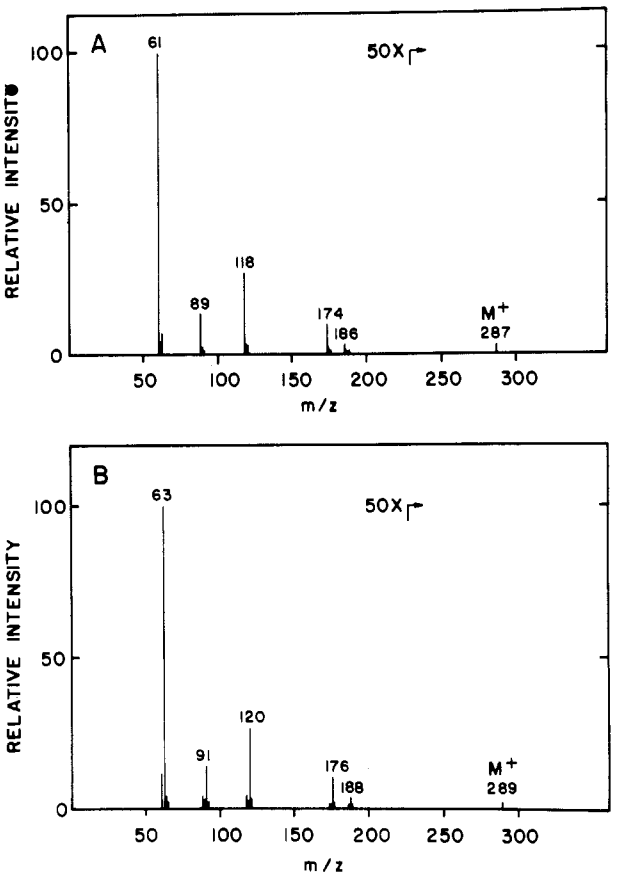

FIGURE 2: Mass spectra of the *N*-(trifluoroacetyl) *n*-butyl ester of *S*-methylcysteine. Cells were grown as described under Experimental Procedures in the presence of (A) 310 μM Na_2SO_4 (unenriched) or (B) 310 μM 90 atom % $Na_2{}^{34}SO_4$. Mass spectra were collected as described in the text.

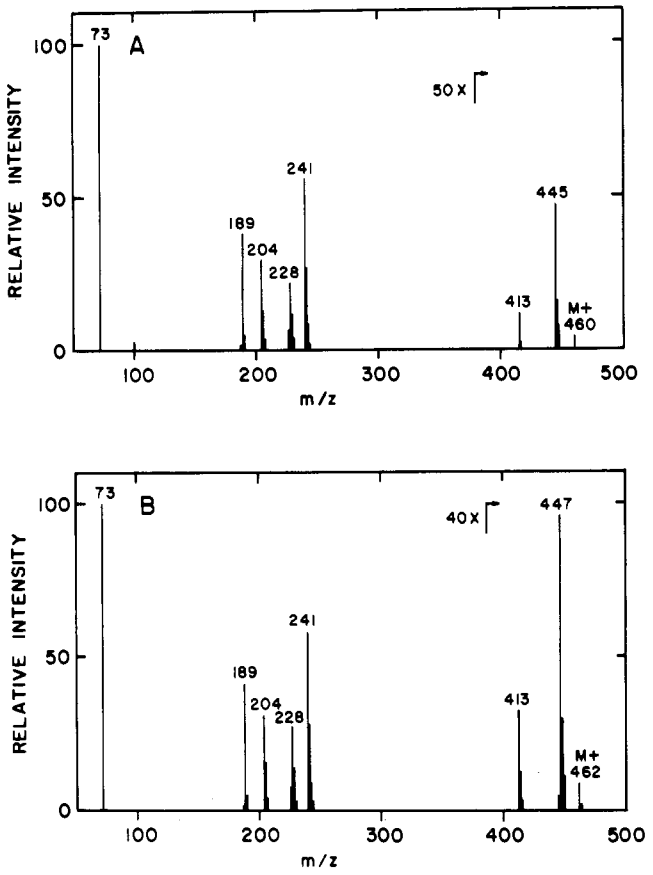

FIGURE 3: Mass spectra of the tris(trimethylsilyl) derivative of biotin. Cells were grown as described under Experimental Procedures in the presence of (A) 310 μM Na_2SO_4 (unenriched) or (B) 310 μM 90 atom % $Na_2{}^{34}SO_4$.

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

expt no.	sulfur source	atom percent excess ^{34}S in biotin	cysteine	methionine
1	50 μM L-[*sulfane*-^{34}S]thiocystine, 150 μM L-methionine	23.0 ± 1.2	23.5 ± 0.6	2.0 ± 0.9
2	40 μM L-[*sulfane*-^{34}S]thiocystine, 30 μM L-cystine, 180 μM L-methionine	18.8 ± 0.8	16.4 ± 1.1	<2
3	20 μM L-[*sulfane*-^{34}S]thiocystine, 60 μM L-cystine, 180 μM L-methionine	10.7 ± 0.7	10.5 ± 0.5	<2
4	2 μM L-[*sulfane*-^{34}S]thiocystine, 87 μM L-cystine, 180 μM L-methionine	<2	<2	<2

[a] *E. coli* hpb λ^- was grown in the presence of the indicated sulfur sources, and values for atom percent excess ^{34}S were obtained as described under Experimental Procedures. Data are expressed as mean ± standard deviation, n = 3–6.

ogenous cystine concentration was increased relative to that of thiocystine, ^{34}S incorporation into biotin was lowered by approximately the amount expected if all sulfur atoms in cystine and thiocystine were equivalent sources of sulfur (e.g., experiment 2 calculated incorporation 22%, actual 18%; experiment 3 calculated incorporation 11%, actual 11%).

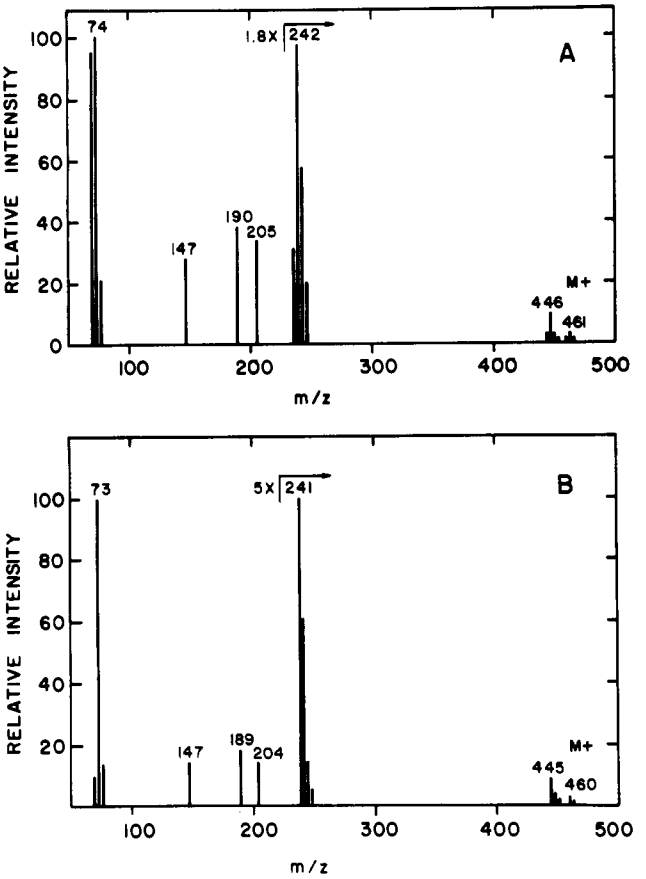

FIGURE 4: Mass spectra of the tris(trimethylsilyl) derivative of biotin isolated from the growth medium of (A) [^{15}N]methionine-supplemented *E. coli* K-12 hpb λ^- cells and from the medium of (B) cells grown without ^{15}N supplement

The decrease in the atom percent excess ^{34}S from that calculated, assuming complete incorporation of ^{34}S from thiocystine into biotin, seen in experiment 1 and again in experiment 2 to a lesser degree (Table II), is probably a consequence of the instability of thiocystine at neutral pH. Szczepkowski & Wood (1967) have shown that at neutral pH, thiocystine slowly decomposes to cystine and elemental sulfur but that this decomposition may be abated by addition of cystine.

The results presented herein are in agreement with our earlier ones (DeMoll & Shive, 1983) in which we showed that [^{35}S]biotin isolated from the supernatant fraction of *E. coli* grown in the presence of L-[^{35}S]cystine had the same specific activity as the cystine supplement and that the radioactive label incorporated into the biotin could not be reduced by addition of methionine to the growth medium. In this study, the use of gas chromatography–mass spectrometry allowed us to examine internal sulfur reservoirs and compare their ^{34}S content with that of the excreted biotin. Consequently, we are now able to present more conclusive evidence of the close relationship of cysteine with the metabolic origin of the sulfur atom in biotin. Whether or not cysteine is the actual, direct donor of sulfur in biotin biosynthesis remains to be shown. This question has proved difficult to study due to the complete loss of the enzyme activity, which converts dethiobiotin to biotin, upon disruption of the cells (Eisenberg, 1975).

Nitrogen Incorporation. The procedures developed for study of the incorporation of isotopic sulfur compounds were applied to the incorporation of [^{15}N]methionine into biotin in *E. coli* to produce the mass spectrum of the trimethylsilyl derivative of biotin shown in Figure 4A. The spectrum of unenriched biotin is shown in Figure 4B. Atom percent excess ^{15}N in-

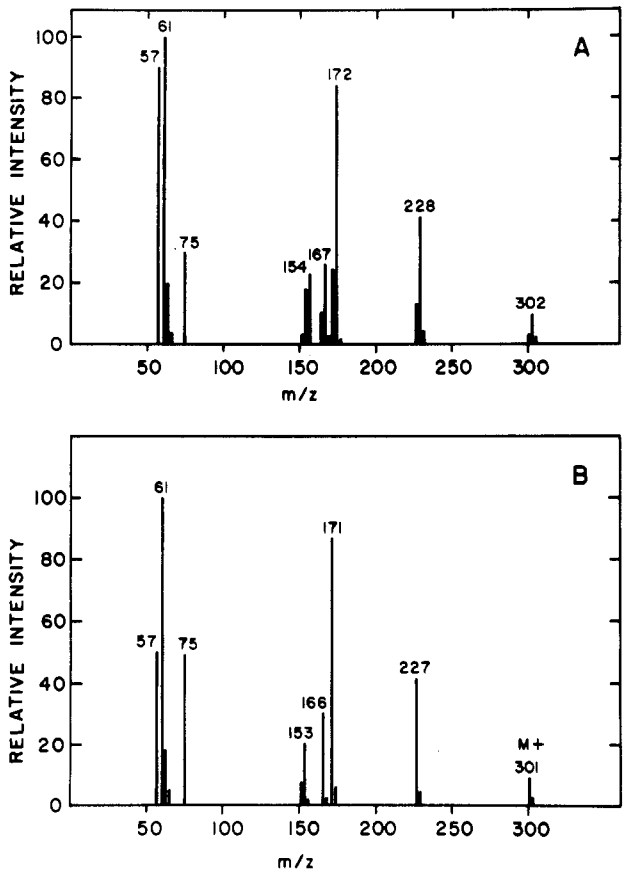

FIGURE 5: Mass spectra of the *n*-butyl ester of *N*-(trifluoroacetyl)-methionine isolated from (A) [^{15}N]methionine-supplemented *E. coli* hpb λ^- cells and from (B) cells grown without ^{15}N supplement.

corporated into biotin was 71.4, indicating a transfer of nitrogen from methionine to biotin without significant dilution by the other amino acids.

This was further confirmed by the examination of the incorporation of ^{15}N into 15 other amino acids isolated from the protein of *E. coli* cells. Of the amino acids incorporated into protein, methionine was labeled with 74.3 atom % excess ^{15}N, and no other amino acid possessed more than 4.3 atom % excess ^{15}N as indicated in Table III. Thus, the atom percent excess ^{15}N incorporated from exogenous methionine into methionine of protein and into one of the nitrogens of biotin was approximately equivalent. The *N*-(trifluoroacetyl) *n*-butyl ester of methionine incorporated into protein of *E. coli* cells grown in the presence of [^{15}N]methionine gave the mass spectrum indicated in Figure 5A. The spectrum of methionine isolated from cells grown with unenriched methionine is shown in Figure 5B. The reduction of the 95 atom % [^{15}N]-methionine to approximately 75 atom % ^{15}N was presumably caused by endogenous methionine biosynthesis, which is reduced by the exogenous methionine supplement but is not entirely eliminated.

Although biotin contains a nitrogen atom in the 1′- as well as the 3′-position, the need to distinguish between these two atoms in the mass spectrum of biotin has been eliminated by the findings of previous investigations. Iwahara et al. (1966), Eisenberg & Star (1968), and Eisenberg & Maseda (1969) have shown that L-alanine condenses with pimeloyl coenzyme A to form 7-keto-8-aminopelargonic acid. The 1′-nitrogen atom of biotin is therefore contributed by L-alanine, and consequently, any significant incorporation of ^{15}N seen in the experiments presented herein must be in the 3′-nitrogen.

Table III: Extent of Incorporation of Nitrogen of Methionine into Biotin and Amino Acids in *E. coli* K-12 hpb λ^- [a]

substance	atom percent excess ^{15}N
excreted from cells	
biotin	71.3 ± 0.7
from incorporation into protein	
methionine	74.3 ± 1.1
proline	4.3 ± 0.6
valine	4.0 ± 0.9
lysine	2.9 ± 0.7
alanine, glycine, threonine, serine, leucine, isoleucine, cysteine, aspartic acid, phenylalanine, tyrosine, glutamic acid, histidine	<2.0

[a] *E. coli* was grown as described under Experimental Procedures. Atom percent excess ^{15}N was calculated according to the procedure of Campbell (1974). Precursor L-methionine was 95 atom % ^{15}N. Data are expressed as mean ± standard deviation, n = 3–6.

Since *S*-adenosylmethionine is known to be essential for the conversion of 7-keto-8-aminopelargonic acid into 7,8-diaminopelargonic acid, it is apparent that this transformation occurs by a direct transfer of the methionine nitrogen of *S*-adenosylmethionine. The pyridoxal phosphate requirement for the conversion indicates transamination but does not exclude other possible mechanisms.

Registry No. Biotin, 58-85-5; dethiobiotin, 533-48-2; nitrogen, 7727-37-9; sulfur, 7704-34-9; methionine, 63-68-3; *S*-adenosylmethionine, 29908-03-0; sulfate, 14808-79-8; thiocystine, 14172-54-4; 7-keto-8-aminopelargonic acid, 4707-58-8; 7,8-diaminopelargonic acid, 21738-21-6; cysteine, 52-90-4; cystine, 56-89-3.

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