Biosynthesis of the Pyrimidine Moiety of Thiamin in *Escherichia coli*: Incorporation of Stable Isotope-Labeled Glycines†

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ABSTRACT: Methods are described for the cleavage, extraction, and subsequent gas chromatographic–mass spectrometric analysis of the pyrimidine moiety of thiamin as 2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine. The methods are of a general nature and can be applied to any system. Using these methods to evaluate the incorporation of ^{13}C-, ^{15}N-, and ^{2}H-labeled glycines into the pyrimidine moiety of thiamin by *Escherichia coli*, we established that the nitrogen and carbon atoms of glycine are incorporated as a unit into the pyrimidine. ^{13}C- and ^{15}N-labeled glycines are incorporated at >60% but deuterium from [2-$^{2}H_2$]glycine was incorporated at only 18%. A detailed analysis of the mass fragmentation pattern of the pyrimidine derivative has established that the glycine nitrogen atom supplies the N-1 of the pyrimidine and that the C-1 and C-2 of the glycine supplies the C-4 and C-6 of the pyrimidine, respectively. This evidence is consistent with the substitution of a C_2 unit between the C-5 and C-4 of the 4-aminoimidazole ribonucleotide precursor during the biosynthesis of the pyrimidine moiety of thiamin in *E. coli*.

At present, the biosynthesis of the pyrimidine moiety of thiamin is poorly understood. Despite its close structural relationship to the pyrimidines found in the nucleic acids, it is clear that it is not biosynthesized by a pathway analogous to that used in pyrimidine biosynthesis (Goldstein & Brown, 1963). Present evidence indicates that 4-aminoimidazole ribonucleotide (AIR), an intermediate in *purine* metabolism, is in fact a precursor for the pyrimidine moiety of thiamin in *E. coli* and *S. typhimurium* (Yura, 1956; Newell & Tucker, 1968).

In this paper, we describe a totally new approach to the study of the biosynthesis of the pyrimidine moiety of thiamin. This is accomplished by the feeding of stable isotope-labeled compounds to *E. coli* and determining by gas chromatography–mass spectrometry their incorporations into a pyrimidine derivative of the biosynthesized thiamin. The results of feeding ^{2}H-, ^{13}C-, and ^{15}N-labeled glycines are presented and discussed.

Experimental Section

Materials

Glycine (99.4 atom % ^{15}N) and sodium formate (91.2 atom % ^{13}C) were obtained from Merck Sharp & Dohme of Canada. Thiamin, thiamin pyrophosphate, and deuterium oxide were obtained from Sigma Chemical Co. (2-Methyl-4-aminopyrimidin-5-yl)methyl chloride hydrochloride was a gift from Dr. G. E. Risinger, Louisiana State University.

Methods

Maintenance and Growth of the Organism. E. coli B was grown on 100 mL of defined liquid medium containing glucose and all of the amino acids except serine as previously described (White, 1978). The labeled glycines were added at 60 mg/100 mL.

Standard Method for the GC–MS Analysis of the Pyrimidine Portion of Thiamin. The fresh *E. coli* cells (1–2 g) were isolated at the end of log phase growth, separated from the growth medium by centrifugation (5000*g*, 20 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 and adjusted to pH 4.6–4.8 with 2 M sodium hydroxide. Centrifugation of this material at 15000*g* for 20 min gave a clear yellow supernatant which was removed and mixed with 0.5 volume of ethanol and 2 mL of ethanethiol. The mixture was then heated at 100 °C for 2.5 h in a sealed tube, after which the ethanol and ethanethiol were removed with a stream of nitrogen. The resulting yellow solution was acidified with 0.2 mL of 6 M HCl and extracted with 5 mL of methylene chloride. After centrifugation to separate the emulsion, the clear upper aqueous layer was removed, treated with 0.2 mL of 6 M sodium hydroxide, and saturated with sodium bicarbonate. This water layer was then extracted three times with 5 mL of methylene chloride, and the methylene chloride extracts were combined and reduced to dryness with a stream of nitrogen. The just visible residue was dissolved in 30 μL of ethyl acetate for GC–MS analysis.

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 Science, Inc.) was used for the analyses described herein. Conditions for the analyses of pyrimidine in *E. coli* extracts were as follows: column, 120 °C; injector, 200 °C; and separator, 260 °C. Under these conditions, the pyrimidine has a retention time of 2 min. All spectra were recorded at 70 eV with a source temperature of 270 °C. Methods for measurement of the isotopic composition of the pyrimidine ions as well as the cellular glycine have been previously described (White, 1978). Metastable peaks were measured from the direct probe mass spectra of crystals of 2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine.

Preparation of 2-Methyl-4-amino-5-[(ethylthio)methyl]-pyrimidine and [methyl-$^{2}H_3$]-2-Methyl-4-amino-5-[(ethylthio)methyl]pyrimidine. (2-Methyl-4-aminopyrimidin-5-yl)methyl chloride hydrochloride (1.94 g) was dissolved in 10 mL of water–methanol (1:1) containing 3.4 g of sodium bicarbonate and 2 mL of ethanethiol. The tube was then sealed and warmed to 70 °C for 1 h with continuous shaking. At the end of the reaction, the methanol and ethanethiol were removed via a stream of nitrogen and the reaction mixture was extracted with methylene chloride. The resulting extract was decolorized with activated carbon and recrystallized three times from benzene–petroleum ether (1:2) to give 0.6 g of white crystals, mp 107–108 °C. This material had ultraviolet absorbance maxima in ethanol at 237 and 268 nm, with extinction coefficients of 4450 and 2520, respectively. An

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additional 0.6 g of crude crystals could be obtained from the mother liquor, mp 98 °C. Fifty milligrams of these crystals was labeled by exchange with DCl in DOAc (from 2 g of acetyl chloride and 3 g of D_2O) for 48 h at room temperature to give [*methyl*-$^2H_3$]-2-methyl-4-amino-5-[(ethylthio)-methyl]pyrimidine.

Preparation of [*amino*-^{15}N]-*2-Methyl-4-amino-5-*[(*ethylthio*)*methyl*]*pyrimidine.* 2-Methyl-4-amino-5-[(ethylthio)-methyl]pyrimidine (580 mg) was hydrolyzed with 5 mL of 6 M HCl at 110 °C for 6 h. The reaction mixture was neutralized with sodium hydroxide and extracted with methylene chloride to give 240 mg of a white solid. After one crystallization from methylene chloride–petroleum ether, 210 mg of 2-methyl-4-hydroxy-5-[(ethylthio)methyl]pyrimidine, mp 130–132 °C, was isolated. One hundred milligrams of this compound was converted into the 4-chloro compound by heating with 1 mL of $POCl_3$ at 78 °C for 3 h (Cline et al., 1937). After removal of the $POCl_3$, the 4-chloropyrimidine was purified by column chromatography to give 120 mg of a colorless oil, M^+ *m/e* 202, 204. To 1.7 mg of this oil dissolved in 0.4 mL of 50% ethanol was added 20 mg of $^{15}NH_4Cl$ and 0.04 mL of 10 M sodium hydroxide. After heating at 140 °C for 24 h the oil was extracted with methylene chloride to give 1 mg of crystals. TLC and GC–MS showed the compound to be pure [*amino*-^{15}N]-2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine.

Preparation of [*6*-^{13}C]-*2-Methyl-4-amino-5-*[(*ethylthio*)-*methyl*]*pyrimidine.* The desired compound was prepared starting with ^{13}C-labeled sodium formate which was converted to the methyl ester and condensed with malononitrile in methanol under basic conditions to give the sodium salt of hydroxymethylenemalononitrile. This was converted to methoxymethylenemalononitrile with dimethyl sulfate in toluene and condensed with acetamidine as described by Grewe (1936). The final product, [6-^{13}C]-2-methyl-4-amino-5-cyanopyrimidine, mp 250 °C, was identical (except for its mass) with a known sample of 2-methyl-4-amino-5-cyanopyrimidine prepared by the condensation of acetamidine with ethoxymethylenemalononitrile (Grewe, 1936). The [6-^{13}C]-2-methyl-4-amino-5-cyanopyrimidine was hydrogenated with palladium on carbon in 3 M HCl to give [6-^{13}C]-2-methyl-4-amino-5-(hydroxymethyl)pyrimidine. This was converted to [6-^{13}C]-2-methyl-4-amino-5-[(ethylthio)-methyl]pyrimidine by acid-catalyzed condensation with ethanethiol.

Results and Discussion

The first step in studying pyrimidine biosynthesis by using mass spectrometry is to develop a method for removing the pyrimidine from the cells and to obtain its mass spectrum. Considering the small concentrations of thiamin in cells and its nonvolatile nature, it was clear that this step would require a derivative which could be prepared from the intact vitamin in a crude state and subsequently separated from the other cellular components so that a mass spectrum of the derivative could be obtained free from other interfering compounds. This separation would also be greatly facilitated if the derivative was volatile and stable enough so that GC–MS could be used to obtain its mass spectrum.

Since it is known that thiamin can be cleaved by the nucleophilic displacement of the thiazole ring with thiols (Bonvicino & Hennessy, 1959), it was felt that cleavage with ethanethiol would give a suitable derivative. The expected product from this reaction, 2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine (I) (see Figure 2), was found to have excellent gas chromatographic properties, both alone and as the trifluoroacetate and trimethylsilyl derivatives, and to partition into organic solvents from the water phase. Based on its ultraviolet absorption at 274 nm, a 0.3 mM solution of I in 0.1 M pH 7 sodium phosphate buffer partitioned 72% into an equal volume of ethyl acetate and 78% into an equal volume of methylene chloride. By saturation of the aqueous layer with sodium bicarbonate, all of I was found to partition into the organic layer. Since methylene chloride may be evaporated to give a residue free of water, it was clearly the solvent of choice for the extraction of the pyrimidine.

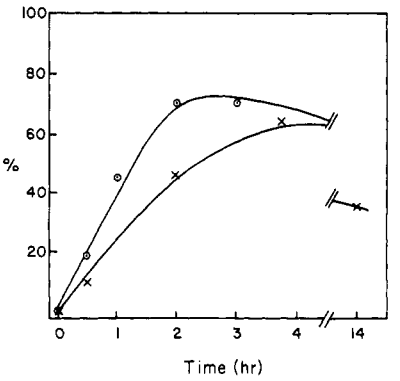

FIGURE 1: Time course for the production of 4-amino-2-methyl-5-[(ethylthio)methyl]pyrimidine generated by the cleavage of thiamin pyrophosphate by ethanethiol. Five-milliliter solutions of thiamin pyrophosphate (10^{-4} M) were mixed with 1 mL of 0.05 M sodium phosphate buffer at pH 6.0, and 2 mL of ethanethiol was added. To one set of tubes (O) 3 mL of ethanol was added, and to another set (X) no ethanol was added. Both sets of tubes were sealed and heated at 100 °C for the indicated times. After cooling and removal of the unreacted ethanethiol, each tube was saturated with sodium bicarbonate and extracted with methylene chloride. The 4-amino-2-methyl-5-[(ethylthio)methyl]pyrimidine was assayed in concentrated methylene chloride extracts by gas chromatography.

Cleavage of the vitamin with ethanethiol was studied next. No reaction between buffer-saturated ethanethiol and thiamin solutions over several hours at room temperature could be observed to give the desired product I. (Assay for the desired product was performed by GC analysis of the methylene chloride extract after saturation of the solution with sodium bicarbonate.) This lack of reaction appears to result from both the low solubility of ethanethiol in water and the apparent inherent slowness of the desired reaction. Both of these factors can be overcome by increasing the temperature.

Quantitating the cleavage of a 0.6 mM thiamin solution in 0.05 M pH 5.0 potassium phosphate buffer saturated with ethanethiol at 60 and 100 °C showed that the maximum recovery of the desired product I (50%) occurred at 100 °C for 4 h. Performing the cleavage under the same conditions of time, temperature, and thiamin concentrations in 0.05 M buffers at the pHs in the indicated buffers (pH 2 glycine; pH 4, 5 acetate; pH 6, 7 phosphate; pH 8 Tris; and pH 9.2 borate) showed that the maximum amount of the product was recovered in the pH 5–6 range. (Note: each of these cleavages was done with enough ethanethiol present so that a liquid phase of ethanethiol was always present, thus allowing the water layer always to be saturated with ethanethiol at any given temperature.)

In order to increase the concentration of ethanethiol in the aqueous phase and thus, hopefully, to increase the yield, ethanol was added to the reaction. As can be seen from Figure 1, the addition of 50% ethanol to the reaction mixture increased not only the yield of I obtained but also reduced the total heating time required for maximal cleavage to ~2.5 h.

Applying these methods to the cleavage of crude thiamin preparations extracted from *E. coli* by dilute acid gave GC

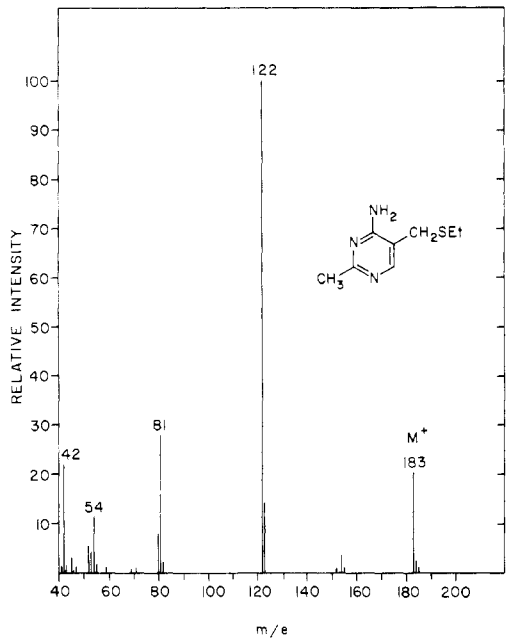

FIGURE 2: Mass spectrum of 4-amino-2-methyl-5-[(ethylthio)-methyl]pyrimidine.

traces in which the desired derivatives were contaminated by several unidentified gas chromatographic peaks. These compounds could, however, be eliminated by taking advantage of the weakly basic nature of the 4-amino group of I. This was done by adjusting the pH of the ethanethiol-cleaved extract with 6 M HCl to less than 1 and extracting with methylene chloride. The impurities were found to extract into the organic layer while protonated I remained in the water layer. After separation of the water phase and saturation with sodium bicarbonate, I could be recovered free from these impurities by extraction with methylene chloride.

By using this purification method, the pH dependency of the cleavage reaction of crude vitamin extracts was studied. The extract was prepared by boiling 23 g of *E. coli* for 20 min in 45 mL of 0.1 M HCl followed by centrifugation at 10000*g*. Five 10-mL portions of this clear yellow extract (pH 1.9) were removed and adjusted to pH 3, 4, 5, 6, or 7 with 2 M sodium hydroxide. After the addition of 5 mL of ethanol and 2.5 mL of ethanethiol to each tube, each was heated at 100 °C for 2.5 h and cooled, and, following the removal of the ethanol and ethanethiol with nitrogen, the reaction mixtures were extracted with methylene chloride as described above. GC analysis gave the following results expressed as percent of maximum recovery: pH 3, 71%; pH 4, 100%; pH 5, 92%; pH 6, 88%; pH 7, 69%. Based on these results all cleavages were done in the range of pH 4–5.

From a consideration of all of the above information, the standard extraction procedure outlined in the experimental section was developed. By using this method, about 20 μg of I could be isolated from the 1.5–2 g of *E. coli* cells produced from 100 mL of growth medium. GC–MS of one-tenth of this extract gave one major peak which gave the same mass spectrum and had the same retention time as a known sample of I. The mass spectrum of this compound is shown in Figure 2.

A detailed analysis of the fragmentation pattern of the pyrimidine was next conducted in order that information on the position of the incorporated isotopes could be obtained. A diagram of the proposed fragmentation of this compound is shown in Figure 3. This scheme is based on the following data: (1) the numerical *m/e* values of the ions observed; (2)

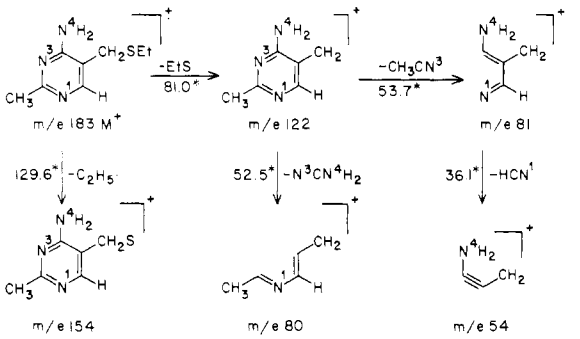

FIGURE 3: Fragmentation of 4-amino-2-methyl-5-[(ethylthio)-methyl]pyrimidine. Starred numbers indicate *m/e* values of metastable ions observed which support the indicated fragmentation. The structure drawn for each ion is to help illustrate the origin of the atoms in the ions from the original structure and does not necessarily represent the true structures of the ions.

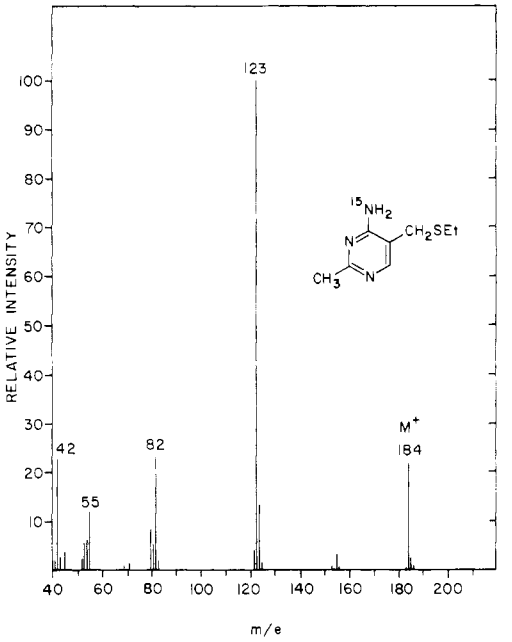

FIGURE 4: Mass spectrum of [*amino*-^{15}N]-4-amino-2-methyl-5-[(ethylthio)methyl]pyrimidine.

the loss or retention of the sulfur isotopes in the ions; (3) the mass spectrum of [*amino*-^{15}N]-2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine; (4) the mass spectrum of [6-^{13}C]-2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine; (5) the mass spectrum of [*methyl*-$^{2}H_3$]-2-methyl-4-amino-5-[(ethylthio)methyl]pyrimidine; (6) the observation of metastable ions for each of the proposed fragmentation pathways; and (7) the comparison with the proposed fragmentation of other simple pyrimidines (Nishiwaki, 1966).

As can be seen in the mass spectrum of the ^{15}N-labeled pyrimidine shown in Figure 4, all of the major ions (*m/e* 184, 123, 82, and 55) have moved up 1 *m/e* over the nonlabeled spectrum. Thus, it can be concluded that the amino nitrogen is not involved in the loss of CH_3CN or HCN from the *m/e* 123 ion. Since the *m/e* 80 ion is in the same position as the nonlabeled molecule, then a fragment containing the amino nitrogen is lost only in generating this fragment. From the difference in the mass of the 122 and 80 ions, this amino nitrogen must be lost along with the N-3 and C-4 atoms of the pyrimidine ring. This is to be compared with the mass spectrum of [6-^{13}C]-2-methyl-4-amino-5-[(ethylthio)-methyl]pyrimidine, shown in Figure 5, where the major ions at *m/e* 184, 123, 82, and 81 have all increased 1 *m/e* but the

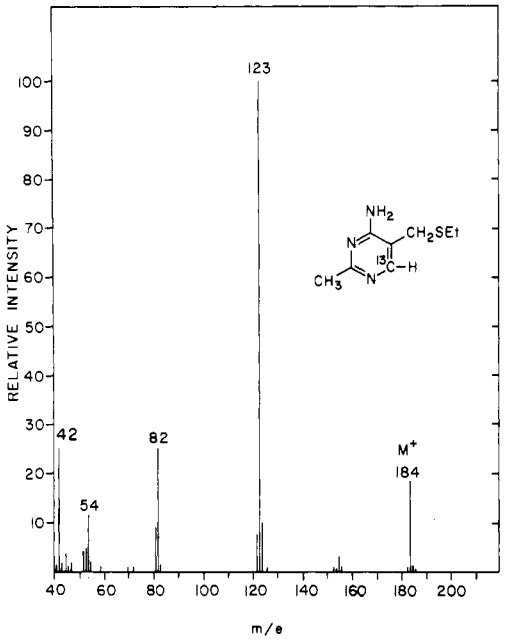

FIGURE 5: Mass spectrum of [6-^{13}C]-4-amino-2-methyl-5-[(ethylthio)methyl]pyrimidine.

m/e 54 ion has remained unchanged. This then shows that the loss of HCN from the *m/e* 81 ion must exclusively involve the loss of this C-6 carbon as well as the N-1 nitrogen of the original pyrimidine. The mass spectrum of the methyl-$^2H_3$-labeled pyrimidine showed an increase of 3 *m/e* in the *m/e* 183, 122, and 80 ions but no change in the *m/e* 81 and *m/e* 54 ions. This indicates that the loss of CD_3CN occurs without scrambling with the other protons in the molecule. Thus, by measuring the isotopic incorporation into the *m/e* 54, 80, and 81 ions and comparing it to the total incorporation measured from the *m/e* 122 or 183 ions, one can calculate both the position and the extent of incorporated labels. Where only ^{15}N-labeled compounds have been incorporated, these measurements will allow for the determination of the extent of nitrogen incorporated into each of the three possible positions. For ^{13}C incorporation, the extent of labeling at C-4 and C-6 of the pyrimidine can be directly determined from the mass spectrum. In addition, total isotope incorporation into the C-2 carbon and its methyl group and the C-5 carbon and its methylene carbon can be determined.

Isotope incorporations are most accurately (±1%) measured from the *m/e* 183 ion because of the absence of $M^+ - 1$ ions and the reduced background in this area of the GC–MS system. The accuracy for the measurement of incorporations into the other ions is somewhat larger and is governed by the intensity of the ions, the column bleed, and interference by other ions. This is particularly troublesome in the *m/e* 80 ion because of the possibility of contamination by a *m/e* 81 − 1 ion. This could lead to a possible error in the measured incorporation of ±10%. The accuracy of the incorporation into the other ions *m/e* 54 and *m/e* 80 is better than ±5%.

The results from the glycine feedings are reported in Table I. The [^{15}N]glycine data clearly show that the N-1 of the pyrimidine ring of thiamin has its origin from the nitrogen of glycine. The mass spectrum from which the data is obtained is shown in Figure 6. The dilution of the ^{15}N from that originally fed is accounted for by the dilution of the nitrogen of the cellular glycine either by transamination, equilibration in the glycine cleavage reaction, or the synthesis of new glycine. This dilution is confirmed by the fact that the ^{15}N of the cellular glycine was found to be 71.3%.

Table I: Incorporation of Labeled Glycines into 2-Methyl-4-amino-5-[(ethylthio)methyl]pyrimidine

	incorpn of label (% of total)				
labeled glycine fed	M^+ *m/e* 183	*m/e* 54	*m/e* 80	*m/e* 81	total cellular Gly
Gly (99 atom % ^{15}N)	74.7	~7.0	63.5	68.7	71.3
[2-^{13}C]Gly (90 atom % ^{13}C)	65.6	~6.0	58.9	67.0	59.0
[1-^{13}C]Gly (90 atom % ^{13}C)	56.2	46.8	<2	56.9	52.5
[2-$^2H_2$]Gly (98 atom % ^{2}H)	18.2	<4.0	19.7	18.8	ND[a]

[a] ND, not determined.

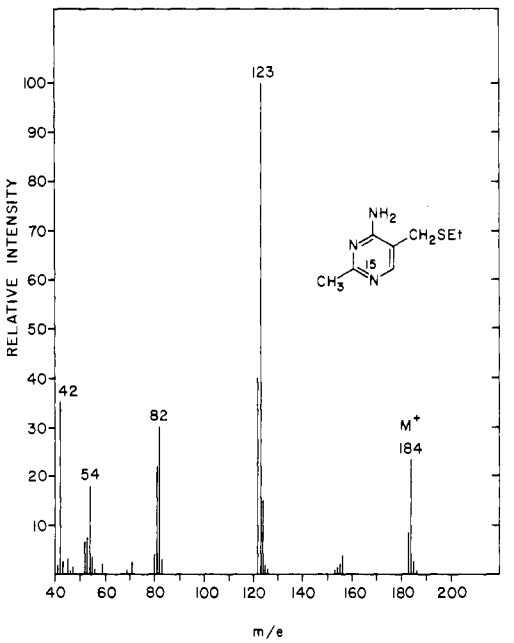

FIGURE 6: Mass spectrum of 4-amino-2-methyl-5-[(ethylthio)-methyl]pyrimidine derived from thiamin synthesized by *E. coli* when grown in the presence of [^{15}N]glycine.

The fragmentation data showing that the glycine nitrogen was incorporated at N-1 was supported in part by the hydrolysis of the ^{15}N-labeled pyrimidine derivative to 2-methyl-4-hydroxy-5-[(ethylthio)methyl]pyrimidine. GC–MS of this derivative showed the same isotopic composition as the starting material, thereby eliminating any ^{15}N incorporation into the amino group.

The results with the [2-^{13}C]glycine (Table I) show that the C-2 atom of glycine is incorporated exclusively into the C-6 position of the pyrimidine. This conclusion was reached because all of the major ions in the mass spectra were found to have increased 1 *m/e*, with the exception of the *m/e* 54 ion. Considering that the fed glycine was only 90 atom % ^{13}C then this glycine accounted for 72.9% of the total glycine incorporated into the pyrimidine. This is about the same as in the ^{15}N case. Again dilution of the isotope by the production of nonlabeled glycine by the cells is confirmed since the cellular glycine has only 59% ^{13}C. The incorporation of the C-2 of glycine into the C-6 of the pyrimidine is supported by the work of Estramareix (1970), who has shown that, by using chemical degradation, the C-2 of glycine supplies the C-6 carbon of the pyrimidine in a mutant of *S. typhimurium*.

[1-^{13}C]Glycine was found to be incorporated at only 56.2% into the pyrimidine or 61.9% when the isotopic content of the

label is considered. This lower incorporation of the C-1 of glycine is believed to result from a selective loss of the C-1 of the glycine during the glycine cleavage reaction. The reversible reaction decomposes glycine to form 1 mol of methylene-H_4folate, ammonia, and CO_2 and has been demonstrated in *E. coli* as well as other bacteria (Pitts & Crosbie, 1962; Newman & Magasanik, 1963). Glycine formed from the reverse of this reaction would thus retain only the C-2 carbon. Considering the difference in the incorporation of the C-1 and C-2 carbons of the glycine, it was calculated that 15.5% of the cellular glycine must have been equilibrated in this reaction. Since the nitrogen would also be lost in this reaction, this then leaves only 9.8% (100 − 74.7 − 15.5%) of the cellular glycine to be produced via transamination or total synthesis. From the isotopes incorporated into the fragment ions of the pyrimidine, it is clear that the C-1 of the labeled glycine is incorporated at C-4 of the pyrimidine ring. This is consistent with the work of Estramareix & Lesieur (1969), who have shown that the C-1 of glycine supplies the C-4 carbon of the pyrimidine in *S. typhimurium*.

Considering that both of the carbons and the nitrogen of glycine are incorporated with no dilution from the metabolic pool of glycine present in the cell, it is clear that all of these atoms must be incorporated as a unit into the pyrimidine. These results are consistent with the work of Newell & Tucker (1968), who have shown that 4-aminoimidazole ribonucleotide is an intermediate in the biosynthesis of the pyrimidine moiety of thiamin in *S. typhimurium*. This follows from the established fact that both carbons and nitrogen of glycine are incorporated as a unit in the biosynthesis of the 4-aminoimidazole portion of the ribonucleotide.

The lack of incorporation of deuterium from the deuterated glycine is also consistent with AIR being an intermediate in pyrimidine biosynthesis. The loss of deuterium can be accounted for by the reversible carboxylation of AIR to CAIR during purine biosynthesis (Litchfield & Shaw, 1971). This reaction would result in the complete loss of the glycine deuterium for each molecule of AIR run through the carboxylation. The fact that some of the glycine deuterium is incorporated into the final C-6 position of the pyrimidine indicates that deuterium on the C-4 of AIR must not be completely lost during its conversion to the pyrimidine.

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Stabilizing Effect of Cholesterol on Phosphatidylcholine Vesicles Observed by Ultrasonic Velocity Measurement†

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ABSTRACT: The temperature dependence of the ultrasonic velocity was measured in sonicated vesicles of dipalmitoylphosphatidylcholine by varying the content of cholesterol. When cholesterol is incorporated, an anomalous dip of the ultrasonic velocity gradually smeared out. At the same time, the ultrasonic velocity of the membrane increased remarkably above 30 °C due to the increase of the bulk modulus by about 15%. On the other hand, the ultrasonic velocity and the bulk modulus decreased below 30 °C. Comparing the cholesterol-incorporated membrane with vesicles of bovine brain sphingomyelin and human erythrocyte membrane, we discuss the role of cholesterol in biological membranes in terms of the stability of the membrane as a barrier.

Cholesterol is one of the major components in biological membranes, yet the role of cholesterol in membranes is not clear enough. Recent investigations by model membranes, however, have elucidated the effects of cholesterol on various physicochemical properties of membranes. The effects of cholesterol may be summarized into two kinds of phenomena. One is the broadening of the phase transition, as shown by thermal analysis (Ladbrooke et al., 1968; Hinz & Sturtevant, 1972; Estep et al., 1978), Raman spectroscopy (Lippert & Peticolas, 1971), and permeability measurements (Blok et al., 1977). Another is the dual effect on the fluidity of membranes (Oldfield & Chapman, 1972a,b; Rothman & Engelman, 1972), that is, the rigidizing effect in the liquid crystalline phase and the fluidizing effect in the gel phase (Lippert & Peticolas, 1971; Kawato et al., 1978). These effects of cholesterol are directly related to the barrier properties of membranes rather than to some specific membrane functions. Therefore, it appears that the biological function of cholesterol is concerned with the stability of the membrane as a barrier (Rothman & Engelman, 1972; Oldfield & Chapman, 1971).

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