

The Reaction Sequence in Ergothioneine Biosynthesis: Hercynine as an Intermediate*

AMIR ASKARI AND DONALD B. MELVILLE

From the Departments of Biochemistry, Cornell University Medical College, New York, New York, and the University of Vermont College of Medicine, Burlington, Vermont

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Ergothioneine can be synthesized by the mold, *Neurospora crassa*, from the amino acids, histidine, methionine, and cysteine (1). The intact histidine molecule is used in the synthesis, and the three methyl groups of the betaine moiety can be derived from methionine by a triple transmethylation process (2). Cysteine appears to be an immediate precursor of the sulfur atom of the thiolimidazole ring. In this paper we present data which establish the sequence of these biosynthetic reactions.

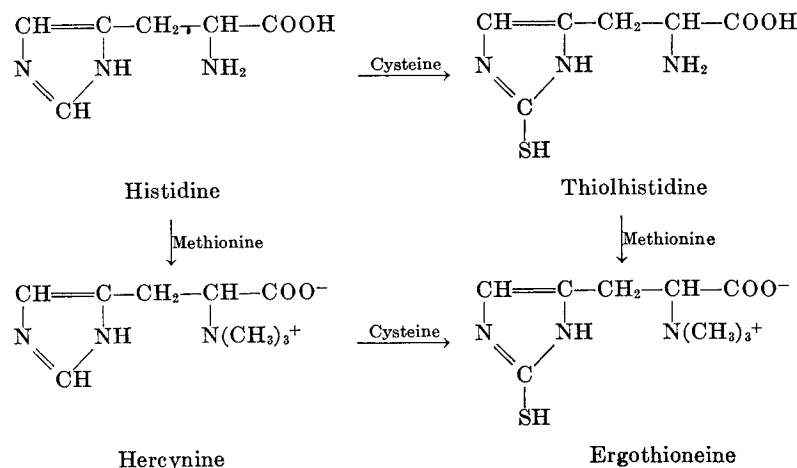
Of the several possible synthetic routes, the most straightforward involve as intermediates either thiohistidine or hercynine, the betaine of histidine, according to the depicted reaction schemes (Scheme 1).

provide data which show that hercynine is an intermediate compound in the biosynthesis of ergothioneine.

EXPERIMENTAL PROCEDURE

Materials—L-Histidine-2-C¹⁴ dihydrochloride was prepared from NaC¹⁴N (4, 5). Sodium formate-C¹⁴ was purchased from Tracerlab, Inc., Boston, Massachusetts. Ergothioneine was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, and was recrystallized from aqueous ethanol.

Methods—*N. crassa* (ATCC 10336, wild type A) was grown in a chemically defined medium (6) in shaken cultures as previously described (1). Ergothioneine was extracted from the dried



SCHEME 1

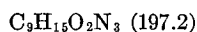
The sequence of reactions involving thiohistidine as an intermediate has not been substantiated by studies which have been done with thiohistidine-2-C¹⁴ (1). The alternative pathway through hercynine has not been explored heretofore. Although hercynine was identified several years ago as a constituent of mushrooms (3), the substance has not been well characterized, and no evidence for its biological relationship to ergothioneine has been adduced. In this paper we describe the preparation of crystalline hercynine, including the C¹⁴-labeled compound, and

mycelium with hot water and was purified by chromatography on alumina (7, 8). Column fractions were analyzed for ergothioneine by the modified Hunter diazo reaction (9). Dried aliquots in steel planchets were examined for radioactivity with a mica window counter.

Preparation of Hercynine—Hercynine was prepared by the oxidation of ergothioneine with ferric chloride (10). A solution of 100 mg of ergothioneine in 0.5 ml of water was mixed with 672 mg of FeCl₃·6H₂O dissolved in 6 ml of water. The mixture was heated under reflux for 30 minutes and then was cooled to room temperature. An aqueous 10% solution of Na₂CO₃ was added, a few drops at a time with centrifugation, until the supernatant solution showed no further precipitation on the addition of Na₂CO₃. The final supernatant solution was removed, and the precipitate was washed twice with 3-ml portions of water. The

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combined solutions were evaporated in a vacuum to dryness. The residue was extracted with three 4-ml portions of hot methanol, and the combined extracts were filtered, made acid to litmus with a few drops of concentrated HCl, refiltered, and evaporated to dryness in a vacuum. The residue was extracted with three 2-ml portions of dimethylformamide, and the extracts were combined, filtered, and evaporated to dryness in a vacuum. This residue was chromatographed on a column of 20 g of alumina with 100 ml of 80% ethanol as the solvent, 5-ml fractions being collected. Aliquots of each were tested with the modified Hunter diazo test for ergothioneine. Those fractions giving a yellow color in the test were combined, the solvent was removed under reduced pressure, and the residue was extracted with three 1-ml portions of methanol. Ether was added to the combined filtered extracts until the solution was turbid. On standing overnight in the refrigerator, the solution deposited rosettes of small rod-shaped crystals which were washed with ether and dried. The crystals weighed 35 mg; an additional 10 mg were obtained from the mother liquors. For analysis the material was recrystallized twice from methanol-ether mixtures. A test for ionic halogen was negative.



Calculated: C 54.82, H 7.67, N 21.31
 Found: C 54.72, H 7.85, N 21.04

Hercynine prepared in this fashion is a colorless substance which is readily soluble in water, methanol, hot ethanol, acetone, and dimethylformamide, but is insoluble in ether, benzene, chloroform, and ethyl acetate. It is hygroscopic, particularly when impure. The compound melts with decomposition at 235–237° (corrected) on the micro melting point stage, with charring above 210°. No absorption occurs in the ultraviolet range at wave lengths longer than 230 m μ . $[\alpha]_D^{25} +51.5^\circ$ ($c = 1.49$ in water).

Preparation of Doubly Labeled Hercynine-C¹⁴—Hercynine labeled with C¹⁴ in both the imidazole ring and the methyl groups was obtained by the degradation of doubly labeled ergothioneine-C¹⁴, which in turn was prepared biosynthetically by growing *N. crassa* in the presence of formate-C¹⁴. To each of 10 flasks, each containing 100 ml of growth medium, were added 5.44 mg (0.08 mc) of sodium formate-C¹⁴. To one of the flasks approximately 0.25 mc of carrier-free sulfuric acid-S³⁵ was also added. *N. crassa* was grown in the media from spore inocula for 96 hours. Each mycelium was then shaken for 24 hours in 100 ml of a sterilized aqueous solution of isotopic formate, or formate and sulfate, in the same concentrations as were used in the growth medium. Each mycelium was then collected on a filter and heated in an autoclave at 15 pounds of pressure for 30 minutes with 30 ml of water. The mycelia were washed twice with small portions of hot water, and the combined extracts and washings were evaporated to dryness in a vacuum after the addition of 40 mg of ergothioneine. The residue was chromatographed on alumina with 75% ethanol-1% formic acid as the solvent. The effluent fractions which contained ergothioneine, as determined by the diazo test, were combined and evaporated to dryness. This material was then treated with ferric chloride as described above for the preparation of hercynine. From the reaction mixture, 23 mg of twice crystallized product were obtained which gave a single diazo-positive (11) and radioactive spot with the same R_F as that shown by hercynine (0.46) after

paper chromatography with *n*-butanol-methanol-benzene-water (1:2:1:1) as the solvent.

A 75- μ g sample of the product, prepared as a thin film for measurement of radioactivity, showed 2180 c.p.m. A 150- μ g sample was added to an aqueous solution of 5 mg of nonisotopic ergothioneine and 5 mg of Na₂SO₄, and the mixture was treated with an excess of bromine-water. The addition of barium chloride yielded barium sulfate, which was collected as a pad on filter paper and showed 9 c.p.m.

Biosynthesis of Ergothioneine from Doubly Labeled Hercynine-C¹⁴—A 48-hour culture of *N. crassa* was washed with sterile water and then was suspended in a sterile solution of 6 mg of the hercynine-C¹⁴ in 100 ml of water. The mixture was shaken for 24 hours, and the mycelium was collected, washed with water, and autoclaved with 35 ml of water containing 10 mg of nonisotopic ergothioneine. The aqueous extract was chromatographed on alumina with 75% ethanol-1% formic acid, and the ergothioneine-containing fractions were combined and chromatographed twice on alumina, each time with 80% ethanol as the solvent. Analysis of aliquots from the effluent fractions from the last column showed excellent correspondence between ergothioneine and radioactivity determinations. These fractions were combined, 20 mg of nonisotopic ergothioneine were added, and the ergothioneine was crystallized from aqueous ethanol to yield 23.6 mg of radioactive product. Recrystallization did not decrease the specific activity. The thrice crystallized compound was used for degradation to thiolurocanic acid and trimethylamine by treatment with hot concentrated NaOH as described previously (1). From 8.8 mg of the ergothioneine there were obtained, after crystallization of the reaction products to constant specific radioactivities, 1.6 mg of thiolurocanic acid and 1.0 mg of trimethylamine chloroplatinate showing 302 c.p.m. and 116 c.p.m., respectively.

In order to compare these radioactivities with that of the doubly labeled hercynine, 150 μ g of the C¹⁴-labeled hercynine were mixed with 12.6 mg of nonisotopic hercynine, and the mixture was subjected to treatment with hot concentrated alkali as described for ergothioneine. Trimethylamine was readily liberated under these conditions and was purified to constant radioactivity as the chloroplatinate. For the isolation of urocanic acid, the alkaline reaction mixture was acidified, evaporated to dryness in a vacuum, and extracted with dimethylformamide. Urocanic acid was precipitated from the filtered extract by the addition of ether and was purified to constant radioactivity by repeated crystallization from dimethylformamide-ether mixtures. The final product was obtained as 1.8 mg of fine needles, micro m.p. 222–226°, with a radioactivity of 276 c.p.m. The trimethylamine chloroplatinate weighed 4.8 mg and showed 362 c.p.m.

The radioactivities of the doubly labeled hercynine-C¹⁴ and the ergothioneine-C¹⁴ synthesized from it by *N. crassa* are compared in Table I.

Competition between Histidine and Hercynine in Biosynthesis of Ergothioneine—The mycelia from two 48-hour growth cultures of *N. crassa* were collected together on a filter and washed with water under sterile conditions. The mycelial pad was divided into two equal parts. One part was placed in a sterile solution of 3.5 mg of histidine-C¹⁴·2HCl (137,000 c.p.m.) in 100 ml of water. The remaining mycelium was placed in a similar solution of 3.5 mg of histidine-C¹⁴·2HCl and 9 mg of nonisotopic hercynine. Each was incubated at 25–27° for 24 hours on a rotary

shaker. Hot water extracts were chromatographed on alumina, first with 75% aqueous ethanol containing 1% formic acid, and then with 80% aqueous ethanol, and the effluent fractions were analyzed for ergothioneine and radioactivity as described earlier. The results are shown in Fig. 1.

The fractions constituting the second radioactive peak in each of the last chromatograms gave the typical magenta-colored diazo test for ergothioneine. The fractions associated with the first radioactive peak gave yellow to orange colors in the diazo test, and these were particularly strong in the fractions from the competition experiment with hercynine and histidine- C^{14} . These latter fractions were combined, 10 mg of nonisotopic hercynine were added, and the hercynine was recovered by crystallization from methanol-ether. The product weighed 8.6 mg and showed 816 c.p.m. The specific radioactivity was unchanged after a second crystallization from methanol-ether. A 7.1-mg sample was decomposed with alkali as described earlier. Crystalline urocanic acid (1.4 mg) was isolated and showed 308 c.p.m. The specific activity did not decrease on recrystallization of the sample.

RESULTS AND DISCUSSION

The preparation of hercynine as the crystalline free base, as described in "Experimental Procedure," has permitted unambiguous studies of its ability to serve as a precursor for the synthesis of ergothioneine by *N. crassa*. The most convenient method of preparing isotopically labeled hercynine for such studies is by oxidative removal of the sulfur atom from correspondingly labeled ergothioneine. The labeled ergothioneine is most readily prepared biosynthetically. In the present work, since it was desirable to employ hercynine labeled in two positions with isotope, formate- C^{14} was used as the precursor for the biosynthesis of the ergothioneine because it had been found earlier (1) that this precursor leads to labeling in both the trimethylammonium radical and the imidazole ring of ergothioneine.

To permit the detection of any ergothioneine- C^{14} which might contaminate the labeled hercynine because of incomplete oxidation of the ergothioneine, sulfate- S^{35} was added to the growth medium in order to label the sulfur atom of ergothioneine. The subsequent treatment of the hercynine with bromine-water, which converts the sulfur of ergothioneine to inorganic sulfate (10), yielded a carrier sulfate fraction which contained negligible radioactivity. This demonstrated that no significant amount of ergothioneine- C^{14} was present in the hercynine- C^{14} .

Initial experiments with hercynine as a possible precursor of ergothioneine yielded negative results. It was established that this was due to the impermeability of the *Neurospora* mycelium to hercynine. Since work in our laboratory by W. Feldman had shown that a similar impermeability of the mycelium toward ergothioneine could be overcome by incubating the mycelium in water rather than in growth medium, the same technique was tried with hercynine and proved to be successful.

When doubly labeled hercynine- C^{14} was incubated in aqueous solution with *Neurospora* mycelium, a significant amount of C^{14} appeared in the ergothioneine which was subsequently isolated from the mycelium. This fact in itself strongly indicates that hercynine is a precursor of ergothioneine. On the other hand, it does not rule out the possibility that only the methyl groups of hercynine are used, or that hercynine is first demethylated to histidine, which is a known precursor of ergothioneine (1, 2).

It was a consideration of these alternative possibilities which dictated the use of hercynine- C^{14} labeled both in the methyl groups and in the imidazole ring, since in either case a preferential loss of C^{14} from one of these positions would occur if only part of the hercynine molecule were incorporated into ergothioneine. A comparison of the isotope distribution in the hercynine- C^{14} used as the precursor with the distribution in the ergothioneine- C^{14} synthesized from it was obtained by decomposition of each with hot alkali, which liberates trimethylamine from both compounds. The specific radioactivities of the trimethylamine and urocanic acid obtained from hercynine, and of the trimethylamine and thiolorocanic acid from ergothioneine, are compared in Table I. Within experimental error, the distribution patterns are identical for the hercynine and ergothioneine. This result shows beyond reasonable doubt that the intact hercynine molecule was incorporated into ergothioneine.

The results of the competition experiment, in which *Neurospora* mycelia were incubated with aqueous solutions of histidine- C^{14} , with and without the addition of nonisotopic hercynine, provide

TABLE I

Distribution of radioactivity in ergothioneine synthesized from hercynine- C^{14} by Neurospora crassa

Wild type *N. crassa* was incubated with hercynine labeled with C^{14} in both the imidazole ring and the methyl groups of the betaine moiety. The resulting ergothioneine was degraded with alkali to thiolorocanic acid and trimethylamine. For comparison of radioactivities, a sample of the hercynine- C^{14} was degraded in similar fashion to urocanic acid and trimethylamine.

Compound	Product	Radio-activity	Ratio of acid to trimethylamine
		c.p.m./mmole	
Hercynine	Urocanic acid	32,100	1.05
	Trimethylamine	30,600	
Ergothioneine	Thiolorocanic acid	21,100	1.06
	Trimethylamine	19,900	

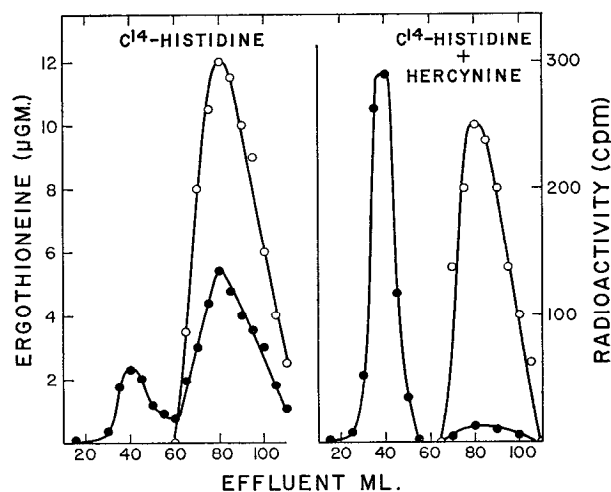


FIG. 1. Distribution of ergothioneine (○) and radioactivity (●) in alumina chromatograms of extracts of *Neurospora crassa*, showing the effect of nonisotopic hercynine on the incorporation of histidine- C^{14} into ergothioneine.

further evidence for the utilization of hercynine in the synthesis of ergothioneine and in addition indicate that hercynine is a normal constituent of *Neurospora crassa* mycelium. A comparison of the distribution of radioactivity and ergothioneine in chromatographic fractions of the mycelial extracts (Fig. 1) shows that the amount of histidine-C¹⁴ converted into ergothioneine is markedly reduced by the presence of nonisotopic hercynine in the incubation mixture, as would be expected if hercynine is an intermediate in the biosynthetic reaction sequence. At the same time, a radioactive peak preceding the ergothioneine peak in the effluent from the columns was greatly increased when nonisotopic hercynine was added to the incubation mixture. The substance responsible for this radioactivity was identified as hercynine by its chromatographic behavior, by its characteristic color in the diazo test, by cocrystallization with authentic hercynine, and by conversion to radioactive urocanic acid on treatment with alkali. The marked increase in radioactivity in hercynine which occurred concomitantly with the decreased amount of radioactivity in ergothioneine can be explained as the dilution and "trapping" by nonisotopic hercynine of hercynine-C¹⁴ synthesized by *Neurospora* from histidine-C¹⁴.

The presence of hercynine in mycelial extracts can be detected most readily by means of the diazo test after the extracts have been chromatographed with aqueous ethanol as the solvent. When formic acid is present in the developing solvent, hercynine and ergothioneine overlap in the effluent, and the yellow or orange color produced by hercynine is obscured by the magenta color given by ergothioneine. A previously unidentified radioactive peak observed earlier (1) in chromatograms prepared from *N. crassa* which had been grown in the presence of histidine-C¹⁴ can, on the basis of the present data, be ascribed to hercynine. Similar peaks have been observed when formate-C¹⁴ or methyl-labeled methionine-C¹⁴ were used as precursors.

The physiological significance of hercynine is unknown. This betaine was isolated from the edible mushroom, *Agaricus campestris*, as the gold salt by Kutscher (3), and from *Boletus edulis* as the picrate and gold salt by Reuter (12). At approximately the same time, Barger and Ewins oxidized ergothioneine with ferric chloride and obtained the picrate of a substance which they considered to be histidine betaine (10). Further work confirmed the identity of the two materials (13, 14). Syntheses of hercynine based on the condensation of α -chloroimidazolepropionic acid with trimethylamine have been described (15, 16), but the free base was not adequately characterized. More recently, Ackermann, List, and Menssen have obtained evidence for the presence of hercynine in the king crab (17), in the red blood cells of cattle and the seminal fluid of the boar (18), and in a snail (19). These workers also detected ergothioneine in the king crab and confirmed its occurrence in cattle red cells and boar seminal fluid. From this association, they have suggested that hercynine serves as a precursor for the biosynthesis of ergothioneine in these species. It should be pointed out that, although lower animals have not been investigated, earlier studies (20) which appeared to demonstrate the synthesis of ergothioneine in the pig have not been confirmed (21, 22); indeed, no evidence has been found for the synthesis of ergothioneine in any higher ani-

mals (5). The possibility that hercynine is formed in the catabolism of ergothioneine in animal tissues has not been investigated. Presently available data suggest that the occurrence of both hercynine and ergothioneine in animal tissues may well be ascribed, at least in part, to dietary constituents which contain both compounds because of a primigenial fungal origin.

SUMMARY

With the aid of C¹⁴-labeled histidine, it has been found that hercynine, the betaine of histidine, is present in *Neurospora crassa* mycelium and is synthesized from histidine by this fungus. Hercynine has been characterized as the crystalline free base. Hercynine which was doubly labeled with C¹⁴, in both the imidazole ring and the trimethylamine moiety, was prepared and was found to be converted to ergothioneine by *N. crassa* without preferential loss of isotope, thus demonstrating that the intact hercynine molecule is incorporated into ergothioneine. The time sequence of biosynthetic reactions involved in the biosynthesis of ergothioneine has therefore been established to be methylation of histidine to form hercynine, followed by sulfhydrylation of hercynine to form ergothioneine.

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