THE BIOSYNTHESIS OF ERGOTHIONEINE*

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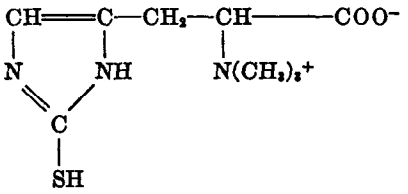
The recent finding that ergothioneine is synthesized by many common fungi (1) has led us to investigate the pathway of biosynthesis. In view of the relatively high concentrations of the substance found in *Neurospora crassa*, this organism was selected for study. The results provide strong evidence to show that ergothioneine (I) is derived from histidine, that cysteine provides the sulfhydryl group, and that the methyl group of



(I)

methionine is the precursor of one or more of the methyl groups of ergothioneine.

EXPERIMENTAL

Materials—L-2-Thiolhistidine and L-histidine, each labeled in the imidazole ring with C^{14}, were prepared from $NaC^{14}N$ by methods similar to those described in the literature (2, 3). The histidine was crystallized and used as the dihydrochloride (4). L-Methionine labeled with C^{14} in the methyl group was synthesized from C^{14}-methanol (5). S^{35}-L-Methionine and S^{35}-L-cystine were purchased from the Schwarz Laboratories, Inc., Mt. Vernon, New York.

Methods—*N. crassa* (ATCC 10336, wild type A) was grown at room temperature in a medium containing sucrose, biotin, and inorganic salts (6). Stationary cultures were prepared by inoculating 50 ml. of medium in a 250 ml. Erlenmeyer flask with a loopful of a spore suspension, and were grown for 7 days. Shaken cultures were grown for 24 hours on a rotary shaker, and they were prepared by inoculating 100 ml. of medium

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in a 250 ml. Erlenmeyer flask with 3 ml. of a heavy suspension of mycelium which had been grown on the shaker in the usual medium to which calcium carbonate (13 gm. per liter) had been added to provide a more uniform suspension. Hot water extracts of the mycelia were prepared and chromatographed on alumina as previously described (1, 7). The effluent fractions were analyzed for ergothioneine by the modified Hunter diazo test (8), and determinations of radioactivity were carried out on dried aliquots by the use of a mica window counter. In many experiments the ergothioneine-containing fractions were rechromatographed to

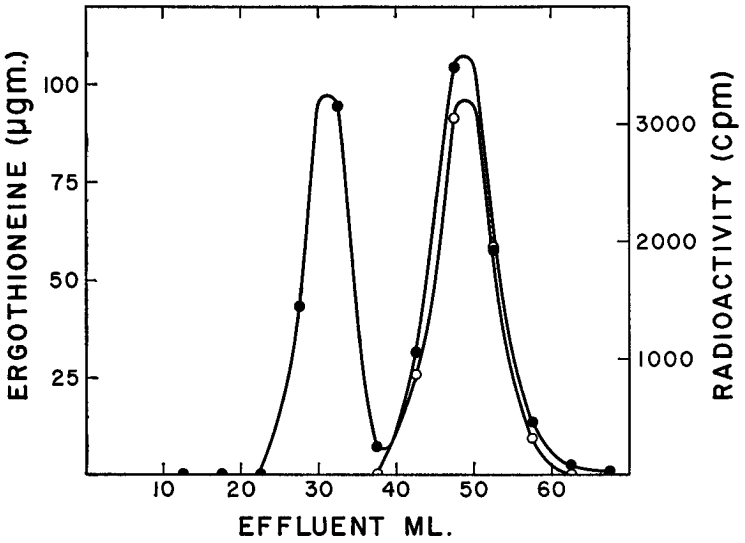

FIG. 1. Distribution of ergothioneine (○) and radioactivity (●) in second alumina chromatogram of aqueous extract of *N. crassa* grown in the presence of histidine-2-C^{14}. Solvent, 80 per cent ethanol.

insure separation of radioactive contaminants from the ergothioneine. A typical chromatogram is shown in Fig. 1.

Histidine As Precursor of Ergothioneine—N. crassa was grown in a shaken culture in the presence of 9.18 mg. of histidine-2-C^{14}. The mycelial extract was chromatographed on alumina with a 1 per cent formic acid-75 per cent ethanol mixture and yielded fractions which showed a radioactive peak in close coincidence with the ergothioneine peak. Because of discrepancies in the specific radioactivities of the ergothioneine-containing fractions, these fractions were combined and rechromatographed on alumina with 80 per cent ethanol as the solvent. A radioactive impurity was separated, but a second radioactive peak showed satisfactory correspondence with the ergothioneine (Fig. 1).

To establish that this radioactivity was in fact due to ergothioneine, and at the same time to determine whether any or all of the activity might be present in the methyl groups of ergothioneine, use was made of the fact that ergothioneine can be degraded to thiolurocanic acid and trimethylamine on treatment with alkali. The fractions which contained ergothioneine (128 γ) were combined and diluted with 14.8 mg. of non-isotopic ergothioneine, and the mixture was crystallized from aqueous ethanol to yield 14.2 mg. of needles. 8.1 mg. of this material were dissolved in 0.5 ml. of 19 N NaOH, and the solution was heated in an oil bath at 130°. The decomposition appeared to be complete in a few minutes. The evolved trimethylamine was swept by means of a stream of N_2 into a trap containing 1 N HCl. The contents of the trap were concentrated *in vacuo*, the residue was dissolved in ethanol, and an alcoholic solution of chloroplatinic acid was added. The precipitate of trimethylamine chloroplatinate weighed 8.0 mg. and showed a radioactivity of 3 c.p.m. The alkaline mixture from the decomposition reaction was acidified to precipitate thiolurocanic acid. The crude product was dissolved in alkali, reprecipitated by the addition of acid, and crystallized twice from aqueous dimethylformamide. The resulting thiolurocanic acid weighed 4.5 mg., with 1400 c.p.m.

For comparison of the specific radioactivities of the ergothioneine and the original C^{14}-histidine, samples of the histidine and the thiolurocanic acid were oxidized by the wet combustion method of Van Slyke *et al.* (9), and barium carbonate pads were prepared. From the observed radioactivities, it was calculated that the specific activity of the histidine was 2.53×10^3 c.p.m. per μmole, and that of the thiolurocanic acid was 2.54×10^3 c.p.m. per μmole.

Origin of Sulfur of Ergothioneine—It has previously been shown that *N. crassa* synthesizes radioactive ergothioneine when grown in the presence of S^{35}-sulfate (1). To determine whether any known sulfur compounds were more immediate precursors of the sulfur of ergothioneine, competition experiments were carried out by growing the mold in the presence of radioactive inorganic sulfate plus various non-isotopic sulfur compounds. Carrier-free S^{35}-sulfate, approximately 3×10^6 c.p.m., and an amount of each competitor sufficient to provide 10 mg. of sulfur were added to the medium. Initial experiments were performed with stationary cultures, but in later ones shaken cultures were used; the results were essentially the same under both conditions. Ergothioneine was isolated chromatographically from the samples of mycelium, and the specific radioactivity was determined in each case. The results of these experiments are listed in Table I.

In view of the effective competition against sulfate shown by the sulfur-

containing amino acids, a further comparison of the precursor abilities of cystine and methionine was made by the use of the S^{35}-labeled amino acids. In these experiments shaken cultures of *N. crassa* were grown in the presence of either 1.02 mg. (1×10^6 c.p.m.) of S^{35}-cystine or 1.30 mg. (1.2×10^6 c.p.m.) of S^{35}-methionine. The ergothioneine isolated from the S^{35}-cystine experiment showed a specific activity of 3.5×10^4 c.p.m. per mg., while that from the experiment with S^{35}-methionine showed 2.1×10^4 c.p.m. per mg.

It was found that the greater effectiveness of cystine compared to methionine as a source of ergothioneine sulfur could be more clearly shown with broken cell preparations of *Neurospora* mycelia. Fresh mycelium

TABLE I

Suppression of S^{35}-Sulfate Incorporation into Neurospora Ergothioneine

Competitor*	Ergothioneine	Suppression
	c.p.m. per mg.	*per cent*
None	23,000	
Thiocyanate	23,800	0
Thioacetamide	20,200	12
Choline sulfate	17,300	25
Thiosulfate	3,400	85
Methionine	3,600	84
Cystine	2,000	91
Cysteine	290	99

* In each case, an amount of competitor sufficient to provide 10 mg. of sulfur was added to the complete medium (6) to which had been added carrier-free S^{35}-sulfate, 3×10^6 c.p.m.

was ground with sand in 0.1 M phosphate buffer, pH 6, the sand was removed, and aliquots of the ground mycelial suspension were shaken with labeled cystine or methionine for 4 hours. Under these conditions cystine proved to be 3 to 5 times more effective than methionine in providing the sulfur of ergothioneine.

To determine whether thiolhistidine might be an intermediate in the biosynthesis of ergothioneine, a stationary culture of *N. crassa* was grown in the presence of 10.5 mg. of 2-thiolhistidine-2-C^{14}. A chromatogram of the mycelial extract in 1 per cent formic acid-75 per cent ethanol showed appreciable radioactivity throughout the effluent fractions, with a total of 1500 c.p.m. in the ergothioneine-containing fractions. However, when these fractions were rechromatographed in 75 per cent ethanol, almost all of the radioactivity was separated from the ergothioneine. The effluent fraction containing the largest amount of ergothioneine (190 γ) showed 17

c.p.m. Part or all of this low activity may be caused by contamination, inasmuch as the correspondence between the determinations of ergothioneine and radioactivity in the effluent fractions was poor.

Methionine As Precursor of Methyl Groups of Ergothioneine—N. crassa was grown in stationary culture in the presence of 9.7 mg. (about 2.5×10^6 c.p.m.) of methionine labeled in the methyl group with C^{14}. The extracted mycelial ergothioneine was chromatographed on alumina, diluted with 2 mg. of non-isotopic ergothioneine, and rechromatographed. The ergothioneine from this column was diluted with 23 mg. of non-isotopic ergothioneine, and the mixture was crystallized four times from aqueous ethanol. The specific radioactivity remained constant after the second crystallization. 10.2 mg. of the material were converted to thiolurocanic acid and trimethylamine by treatment with alkali as previously described. The purified thiolurocanic acid weighed 3.7 mg., with 4 c.p.m., and the trimethylamine chloroplatinate 9.5 mg., with 3770 c.p.m.

DISCUSSION

The foregoing data appear to account completely for the biosynthesis of the ergothioneine molecule as originating in the amino acids histidine, cysteine, and methionine.

The finding that histidine is a precursor is not surprising in view of the similarity in structure of histidine and ergothioneine. It is of interest that C^{14}-histidine was converted to ergothioneine without dilution of radioactivity. This indicates that the synthesis of ergothioneine from endogenous histidine was completely suppressed by the added C^{14}-histidine. It further suggests that the imidazole ring of histidine remains intact in the conversion to ergothioneine. Should the ring be opened at the amidine carbon atom during the introduction of the sulfhydryl group, it might be expected that at least part of this labeled carbon atom would be lost. The fact that thiocyanate was inactive as a precursor of the sulfhydryl group (Table I) is further evidence that the thiolimidazole ring system is not synthesized biologically in a manner comparable to the laboratory synthesis of thiolhistidine from histidine, in which the amidine carbon atom is removed from the imidazole ring and replaced by the carbon atom of thiocyanate.

The inability of thiolhistidine to serve effectively as a precursor of ergothioneine in *Neurospora* cannot readily be ascribed to a possible impermeability of the cells to the added C^{14}-thiolhistidine, since appreciable amounts of radioactivity were detected in crude extracts of the washed cells. It therefore seems probable that thiolhistidine is not an intermediate in ergothioneine formation in *Neurospora*, and consequently suggests that the introduction of methyl groups into histidine precedes the

formation of the sulfhydryl group. The most likely intermediate is hercynine, the betaine of histidine, which is known to occur in some fungi.

The studies on the origin of the sulfur of ergothioneine by means of competition experiments (Table I) demonstrate that thiosulfate and the sulfur-containing amino acids are efficient precursors of the sulfhydryl group. Thiosulfate probably owes its effectiveness to a ready conversion to cysteine or cystine, since in *Escherichia coli* it appears to be an immediate precursor of these amino acids (10). The slight superiority of cystine over methionine which is indicated in Table I is substantiated by the experiments with S^{35}-labeled cystine and methionine, both with intact mycelia and with broken cell preparations. Sodium sulfide was found to be too toxic to test adequately; however, when thioacetamide was used as a source of hydrogen sulfide, negligible competition with sulfate was observed. The most effective precursor which we have tested is cysteine. Although this substance is somewhat toxic to *N. crassa*, it served as an excellent source for ergothioneine sulfur, with almost complete suppression of inorganic sulfate incorporation (Table I). It seems probable that cysteine is an immediate precursor of the sulfhydryl group of ergothioneine, possibly through the formation of a thio ether between cysteine and hercynine.

The conversion of C^{14}-methyl-labeled methionine to ergothioneine labeled almost exclusively in the methyl groups suggests the possibility of a transmethylation reaction but does not prove it, since intermediary conversion of the methyl group of methionine to "formaldehyde" or "formate" may be occurring. The possibility that free formate might be involved was tested by growing *N. crassa* in the presence of C^{14}-labeled formate. The isolated ergothioneine was converted to thiolurocanic acid and trimethylamine as described under "Experimental." Both showed appreciable radioactivity; in fact, the thiolurocanic acid was more active than the trimethylamine, with 3.2×10^4 c.p.m. per mmole compared to 1.4×10^4 c.p.m. per mmole of trimethylamine. Inasmuch as the thiolurocanic acid from the experiment with C^{14}-methionine was almost devoid of radioactivity, these results suggest that free formate is not produced during the transfer of the methyl carbon of methionine to the methyl carbons of ergothioneine, and to that extent support the supposition that a transmethylation reaction may be involved.

Although the studies described in this paper have been carried out with *N. crassa*, it seems likely that the results can be applied to other fungi which synthesize ergothioneine. In this regard it is of interest that the preliminary studies of ergothioneine synthesis by *Claviceps purpurea* reported by Heath and Wildy (11, 12) are in agreement with the work outlined in this paper.

SUMMARY

The pathway of biosynthesis of ergothioneine by *Neurospora crassa* has been studied by growing the fungus in the presence of C^{14}- and S^{35}-labeled compounds. That ergothioneine is derived from histidine was shown by the formation of C^{14}-ergothioneine from L-histidine-2-C^{14} without dilution of the radioactivity. Thiolhistidine-2-C^{14}, on the other hand, does not serve as a precursor to any significant extent. The sulfhydryl group of ergothioneine is readily derived from thiosulfate and the sulfur-containing amino acids; cysteine is the most effective precursor as judged by its ability to suppress the incorporation of S^{35}-sulfate into ergothioneine. When *N. crassa* is grown in the presence of methyl-labeled C^{14}-methionine, C^{14}-ergothioneine is formed, and the isotope is present almost exclusively in the methyl groups of the ergothioneine.

BIBLIOGRAPHY

1. Melville, D. B., Genghof, D. S., Inamine, E., and Kovalenko, V., *J. Biol. Chem.*, **223**, 9 (1956).
2. Ashley, J. N., and Harington, C. R., *J. Chem. Soc.*, 2586 (1930).
3. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **187**, 839 (1950).
4. Melville, D. B., Horner, W. H., Otken, C. C., and Ludwig, M. L., *J. Biol. Chem.*, **213**, 61 (1955).
5. Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169**, 419 (1947).
6. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943).
7. Melville, D. B., Horner, W. H., and Lubschez, R., *J. Biol. Chem.*, **206**, 221 (1954).
8. Melville, D. B., and Lubschez, R., *J. Biol. Chem.*, **200**, 275 (1953).
9. Van Slyke, D. D., Plazin, J., and Weisiger, J. R., *J. Biol. Chem.*, **191**, 299 (1951).
10. Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T., and Britten, R. J., *Carnegie Inst. Washington, Pub. No. 607* (1955).
11. Heath, H., and Wildy, J., *Biochem. J.*, **63**, 1P (1956).
12. Wildy, J., and Heath, H., *Biochem. J.*, **63**, 32P (1956).