Biosynthesis of Ergothioneine by *Claviceps purpurea*

2. INCORPORATION OF [^{35}S]METHIONINE AND THE NON-UTILIZATION OF [2(RING)-^{14}C]HISTAMINE*

By JENNIFER WILDY and H. HEATH
Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

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The incorporation of [2-^{14}C]acetate into ergothioneine by *Claviceps purpurea* has been previously reported by Heath & Wildy (1956*a*, *b*). This work showed that there was a close relationship between histidine and ergothioneine biosynthesis. Histidine might be converted directly into ergothioneine in the fungus or alternatively ergothioneine might be derived from a compound closely related structurally to histidine.

Since histamine occurs in ergot (Barger & Dale, 1910) and its conversion into ergothioneine could be envisaged, it was decided to test this substance as an ergothioneine precursor in our system, as [2(ring)-^{14}C]histamine was immediately available and [2(ring)-^{14}C]histidine was not available to us at the time. Studies with [2-^{14}C]histidine will be reported later. If ergothioneine is formed directly from histidine, then in order that the [2-^{14}C]histamine should be incorporated into the ergothioneine, it would be necessary for it to be first carboxylated and this would result in the general pool of histidine in the fungus being labelled. If, on the other hand, ergothioneine is formed from histidine by the partial degradation of the side chain, such as to imidazole-ethanol, which could also be formed by deamination of histamine, followed by a condensation to yield the betaine of ergothioneine, then labelled ergothioneine could be derived from [2-^{14}C]histamine without the formation of labelled histidine. The same result would be obtained if histamine derived by decarboxylation of histidine were methylated and subsequently recarboxylated.

Sulphate can provide the sulphur for ergothioneine in this system (Heath & Wildy, 1956*b*) but nothing is known of the immediate sulphur precursors. In order to elucidate further this aspect of the problem, the incorporation of [^{35}S]methionine was studied.

Some of these results have been presented in a preliminary form (Wildy & Heath, 1956).

EXPERIMENTAL

Organism. The culture of *Claviceps purpurea* no. 44613 was originally obtained from the Commonwealth Mycological Institute, Kew, London, and was maintained as described previously (Heath & Wildy, 1956*b*).

[2(*ring*)-^{14}C]*Histamine and* DL-[^{35}S]*methionine.* These were obtained from the Radiochemical Centre, Amersham. The activity of the histamine dihydrochloride was 4·7 mc/m-mole. At the commencement of the methionine experiment 5 mg. of DL-methionine gave $1{\cdot}24 \times 10^7$ counts/min. counted at infinite thinness with a thin mica end-window Geiger tube.

The following materials and methods used have been previously described (Heath & Wildy, 1956*b*): activated alumina; Zeo-Karb 225; Oxoid Membrane Filters; sterilization; culture media; preparation of inoculum; detection and estimation of ergothioneine.

Growth of Claviceps purpurea *on a medium containing* [2-^{14}C]*histamine.* Five 120 ml. shake cultures were grown in 350 ml. Erlenmeyer flasks. Two of these were controls containing only the standard mannitol–ammonium succinate medium; one flask was supplemented with 0·1 mc of [2-^{14}C]histamine dihydrochloride (3·9 mg.); 0·1 ml. of this medium was removed before sterilization in order to determine the total activity present, as measured by our apparatus; the remaining two flasks were each supplemented with 3·9 mg. of unlabelled histamine dihydrochloride. All were inoculated with 1 ml. of conidial suspension and the cultures were grown simultaneously at room temperature for 10 days.

The total growth (mycelium and conidia) was harvested separately from each flask by filtration on Oxoid membranes and washing with water. The filtrate and washings from the [2-^{14}C]histamine flask were made up to 250 ml. and a portion was used to determine the activity remaining in the culture broth.

Ergothioneine was estimated in the control growth to ensure that the fungus was still producing ergothioneine (at one stage in this work the fungus failed to produce ergothioneine and it was always necessary to check with control growth). In one histamine control, estimation of ergothioneine was found to be impracticable owing to interference with the Hunter-colour reaction by other diazo-reacting compounds, which were not separated by the alumina chromatography. The presence of ergothioneine was definitely established by paper chromatography of the eluate from the alumina column. From the appearance of the chromatogram it was apparent that the usual amount of ergothioneine was present.

Isolation of ergothioneine from the [2-^{14}C]*histamine culture.* L-Ergothioneine hydrochloride (90 mg.) was added to the total growth obtained from the [2-^{14}C]histamine experiment, which was then extracted three times with water (total vol. 40 ml.) by immersion in boiling water. The residue was then extracted twice by boiling with water (total vol. 40 ml.). The

* Part 1: Heath & Wildy (1956*b*).

combined extracts were deproteinized and chromatographed on Zeo-Karb 225 (4½% cross-linked) and alumina as described (Heath & Wildy, 1956*b*) for the isolation of [^{14}C]ergothioneine from the growth of *C. purpurea* on [2-^{14}C]acetate. In the present case, however, the ergothioneine-containing fractions from the alumina column were taken to dryness and rechromatographed on a second alumina column and finally on a Zeo-Karb 225 column. The resulting ergothioneine, after extraction with ethanol, weighed 63·5 mg. It was recrystallized seven times and its radioactivity measured after each recrystallization.

Isolation of histidine from the [2-^{14}C]*histamine culture.* The residue of the fungus after aqueous extraction was hydrolysed by refluxing in 6N-HCl for 20 hr., evaporated to dryness and finally dried in a vacuum desiccator over NaOH. The residue was dissolved in water and filtered. One-dimensional chromatograms, run in butanol–acetic acid, showed that a general distribution of amino acids, including histidine, was present. Also, a radioactive spot with R_F slightly greater than histidine was detected. The main bulk of the filtrate was brought to pH 4 with 2N-NaOH, and L-histidine hydrochloride monohydrate (250 mg.) was added, followed by saturated ethanolic $HgCl_2$ (10 ml.); the pH was adjusted to 7·2 with saturated $NaHCO_3$ to precipitate the mercury complex, and the mixture was allowed to stand at 4° for 1 hr. The precipitate was filtered off, washed with water and decomposed with H_2S. The HgS was removed by filtration, the filtrate was concentrated to 25 ml. and 3:4-dichlorobenzenesulphonic acid (1 g.) was added to the hot solution. After standing overnight at 4° the crystals were filtered off and washed with ice-cold water; wt. 450 mg. It was recrystallized five times and the radioactivity of the crystals was measured each time.

Labelled compounds remaining in culture broth after growth of Claviceps purpurea *on* [2-^{14}C]*histamine.* The filtrate and washings obtained during the harvesting of the fungus grown on [2-^{14}C]histamine were passed through a 15 cm. × 0·9 cm. column of the H^+ form of Zeo-Karb 225, 4½% cross-linked, 100–150 mesh. All the radioactivity was retained by the column which, after washing with water, was eluted with 0·1N-NH_3 soln. and the radioactive fractions were combined and evaporated to dryness. The residue was dissolved in 0·4 ml. of water and 10 µl. portions were subjected to ascending chromatography in *n*-butanol saturated with 6N-NH_3 soln. (a solvent which effectively separates histamine from histidine; Urbach, 1949). The chromatograms were developed with ninhydrin, Pauly diazo reagent and dichloroquinonechloroimide, and scanned with an end-window Geiger counter.

A volume (50 µl.) of the solution was mixed with 50 µl. of 12N-HCl and hydrolysed by heating at 100° for 24 hr. in a sealed capillary. The dried residue was dissolved in water and chromatographed in ascending butanol–NH_3 soln. and descending butanol–acetic acid.

Growth of Claviceps purpurea *on a medium containing* [^{35}S]*methionine.* DL[^{35}S]Methionine (5 mg.) was added to 120 ml. of the standard mannitol–ammonium succinate medium in a 350 ml. Erlenmeyer flask, inoculated with 1 ml. of conidial suspension and grown for 10 days. Ergothioneine was isolated from the total growth by extraction with water, ethanol deproteinization and alumina chromatography. The [^{35}S]ergothioneine thus obtained was crystallized to constant activity after conversion into ergothioneine tartrate.

RESULTS

The activity at infinite thinness of the [2-^{14}C]-histamine medium before growth was $1·58 \times 10^7$ counts/min. and after growth the culture broth gave $1·04 \times 10^7$ counts/min.; hence 66% of the isotope was not incorporated into the total growth.

There was no incorporation of isotope into either the isolated histidine or ergothioneine, but the metabolites of histamine were not easily separated from either histidine or ergothionine either on ion-exchange or alumina columns. This separation was achieved only by repeated crystallizations, as shown in Table 1.

The results of scanning the paper chromatograms showed that activity was associated only with diazo-reacting compounds which were still highly labelled, giving counts on the chromatogram of the order of 3000–6000 counts/min. Free histamine was no longer present in the culture broth; after hydrolysis, however, radioactive histamine was detected, probably derived from an acylated histamine. The histidine spot was not radioactive, showing that carboxylation of histamine to histidine had not occurred. One very radioactive spot was identical with imidazoleacetic acid, both in butanol–ammonia and butanol–acetic acid. From radioautographs of the chromatograms three other diazo-reacting metabolites of histamine were detected. No radioactivity was associated with ninhydrin-reacting substances. It appears, therefore, that the metabolism of histamine by this fungus brings about the degradation of the side chain but does not break down the imidazole ring.

The ergothioneine isolated from the culture of *C. purpurea* grown on [^{35}S]methionine had an activity of 3542 counts/min. for 1 cm.^{2} at infinite

Table 1. *Decrease in radioactivity of ergothioneine and histidine bis-3:4-dichlorobenzenesulphonate isolated from a culture of* Claviceps purpurea *grown on a medium containing* [2-^{14}C]*histamine, on crystallization from aqueous ethanol and water respectively*

Activities are given as counts/min., at infinite thickness of 1 cm.^{2} areas.

	Activities	
Crystallization	Histidine bis-3:4-dichloro-benzenesulphonate	Ergothioneine
1st	3315	1274
2nd	1264	610
3rd	400	205
4th	147	140
5th	50	63
6th	8	39
7th	—	32
8th	—	20

thickness after crystallization, to constant radioactivity, from water as the tartrate. Scanning of paper chromatograms revealed only one radioactive spot, which coincided with ergothioneine.

DISCUSSION

As can be seen from the results, neither the ergothioneine nor the histidine isolated from a culture of *Claviceps purpurea* grown on a medium containing [2-^{14}C]histamine was labelled. In previous communications (Heath & Wildy, 1956*a*, *b*) it has been shown by the incorporation of [2-^{14}C]acetate that there is a direct relationship between the biosynthesis of ergothioneine and histidine in this system. It is obvious from the present results that the carboxylation of histamine does not take place. This is not unexpected since the carboxylation of amines to yield amino acids is not a normal biosynthetic pathway, although certain examples of the reversibility of amino acid decarboxylases have been reported (Hanke & Siddiqi, 1950). Although primary amines such as aminoethanol (Horowitz, 1946), noradrenaline (Bülbring, 1949) and tyramine (Leete, Kirkwood & Marion, 1952) have been shown to be methylated in biochemical processes, the possibility that the betaine of ergothioneine is formed by the methylation of histamine followed by carboxylation is ruled out by the above results. The methylation of amino acids, on the other hand, is not a normal biosynthetic process, but some method must exist for the methylation by which the betaine structure of ergothioneine is built up. By analogy with the biosynthesis of betaine, which is formed by the methylation of aminoethanol to give choline with subsequent oxidation to yield betaine and not by the direct methylation of glycine, it might be expected that ergothioneine would be formed by the introduction of methyl groups at the histidinol stage with subsequent oxidation to the betaine. Such a pathway, moreover, would be consistent with our earlier results which show an identical pattern of labelling in both histidine and ergothioneine derived from [2-^{14}C]acetate.

The present work has also thrown light on the metabolism of the imidazole-ring system by this fungus, because the activity remaining in the culture medium was associated with imidazole compounds and there appeared to be no degradation of the imidazole ring to yield labelled fragments. There was no detectable radioactivity in any of the amino acids, and the fraction of the culture broth not absorbed on the Zeo-Karb 225 column, which would contain the carboxylic acids (including formate), was completely inactive. Free histamine no longer occurred, although some could be liberated on acid hydrolysis which was probably derived from an acylated histamine such as acetylhistamine. Oxidation of the side chain had occurred, as shown by the presence of imidazoleacetic acid and the other radioactive diazo-reacting substances. Recently Karjala, Turnquest & Schayer (1956) have shown that the metabolites of histamine in mouse urine consist of 1-methylimidazole-4-acetic acid, 1-methylimidazole-5-acetic acid and 1-ribosylimidazole-4(5)-acetic acid, as well as imidazoleacetic acid. The fission of the imidazole ring of imidazoleacetic acid to yield formylaspartic acid, as shown to occur in *Pseudomonas* by Hayaishi (1955), did not appear to take place in this fungus.

Claviceps purpurea has been shown to be capable of utilizing the sulphur of methionine for the synthesis of ergothioneine. The actual pathway of incorporation is being further studied to determine the direct sulphur precursor, as these results do not necessarily show that methionine itself is a direct sulphur donor.

SUMMARY

1. [2-^{14}C]Histamine was not incorporated into either ergothioneine or histidine by *Claviceps purpurea*.

2. The imidazole ring of histamine was not degraded by this fungus and the residual activity detected was associated only with imidazole compounds. No activity was present in either amino or carboxylic acids.

3. [^{35}S]Ergothioneine was isolated from a culture of *Claviceps purpurea* grown on a medium containing [^{35}S]methionine.

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