Biosynthesis of Ergothioneine by *Claviceps purpurea*

3. THE INCORPORATION OF LABELLED HISTIDINE*

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Degradation of ergothioneine and histidine isolated from cultures of *Claviceps purpurea* grown in the presence of [2-^{14}C]acetate has revealed a similar distribution of labelling in the six-carbon structure common to both these compounds (Heath & Wildy, 1956*a*, *b*). These results indicated that this fungus forms ergothioneine by methylation and sulphuration of histidine.

Subsequent work (Wildy & Heath, 1956, 1957) revealed that [2(ring)-^{14}C]histamine would not serve as a precursor of the imidazole nucleus of either histidine or ergothioneine. Various metabolites of histamine, imidazoles possessing modified side chains, also failed to provide the requirements of an ergothioneine precursor.

In order to confirm that histidine was a direct precursor of ergothioneine, experiments were carried out with histidine labelled both in the side chain and the imidazole ring. A preliminary report of this work has already been published (Heath & Wildy, 1957).

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*).

DL-[α-^{14}C]*Histidine.* This was obtained from Tracer Lab, Boston (activity 0·5 mc/m-mole).

L-[2(*ring*)-^{14}C]*Histidine.* This was obtained from the Radiochemical Centre, Amersham (activity 4 mc/m-mole).

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

Radioautography of chromatograms. Two-dimensional chromatograms were prepared on 10 in. squares of Whatman no. 4 paper by the frame technique of Datta, Dent & Harris (1950). A glass and polythene tank assembly was used; the tank and polythene-coated trough were purchased from Aimer Products Ltd., London, N.W. 1, and the all-polythene frame was made by Mr P. R. E. Wallace of this Department. The radioactive solution (10–30 μl.) was applied to the paper, the dried spot was counted with a mica end-window Geiger tube and the time required for exposure of the subsequently developed chromatogram to the X-ray film was calculated on the basis of this activity. Under our experimental conditions of counting, an exposure equivalent to at least $1{\cdot}5 \times 10^6$ total counts was found to give a satisfactory radioautograph (e.g. a spot giving 1050 counts/min. required a 24 hr. exposure).

The chromatograms were developed with phenol (80% aq. soln.) in the presence of aq. 0·1 N-NH_3 soln., followed by *n*-butanol–acetic acid–water (60:15:25) and then dried in air until no odour of solvent remained. An identification number was written on the edge, and the corners of the square were marked with a radioactive ink so that the paper chromatogram could be subsequently superimposed on the radioautograph. Each chromatogram was then placed in contact with a 10 in. × 12 in. sheet of Kodirex Increased Speed X-ray Film (Kodak Ltd., Kingsway, London, W.C. 2) and left for the required time. The film was then developed for 5 min. at 60° F. in a solution consisting of: *p*-methylaminophenol sulphate (Metol), 2·2 g.; quinol, 8·8 g.; $Na_2SO_3,7H_2O$, 144 g.; $Na_2CO_3,10H_2O$, 130 g.; KBr, 4 g.; water to 1 l. The film was then washed and fixed in a solution of 25% $Na_2S_2O_3,5H_2O$. The paper chromatograms were then treated with ninhydrin.

Growth of C. purpurea *on a medium containing* [α-^{14}C]-*histidine.* DL-[α-^{14}C]Histidine (0·1 mc, 30·4 mg.) was added to 600 ml. of the mannitol–ammonium succinate medium which was then distributed between five 350 ml. Erlenmeyer flasks. Four control cultures were also grown, two containing the basic medium and two supplemented with 6·2 mg. of histidine. The pH of the medium before growth was 5·8. After sterilization the cultures were grown on a rotary shaker for 12 days and were then harvested by filtration through Oxoid Membrane Filters and washed with water; the five cultures containing labelled histidine were harvested together and the four controls separately. The pH of the culture filtrate was 7·6.

Estimation of activity in the culture medium before and after growth. A portion (0·1 ml.) was taken from the culture medium before growth; it was evaporated to dryness in a small Quickfit flask and totally oxidized, the CO_2 being collected as $BaCO_3$ which was plated and counted at infinite thickness in a polythene planchet. A portion (0·2 ml.) of the culture filtrate was evaporated to dryness and oxidized in the same way. The culture filtrate was passed through a column of Zeo-Karb 225, H^+ form; 0·1 ml. of the culture filtrate and 0·1 ml. of the eluate from the column were estimated for activity by counting as infinitely thin film.

Ergothioneine was either estimated or detected chromatographically in the control cultures in order to ensure that the fungus was still producing ergothioneine in the presence of added histidine.

* Part 2: Wildy & Heath (1957).

Isolation of erogthioneine from the [α-^{14}C]*histidine culture.* L-Ergothioneine hydrochloride (90 mg.) was added to the total growth, which was then extracted three times with hot water (total vol. 120 ml.). The residue was then extracted twice with boiling water (total vol. 140 ml.) and filtered through an Oxoid Membrane. The combined extracts were deproteinized and chromatographed on Zeo-Karb 225 and alumina as described previously (Heath & Wildy, 1956*b*) for the isolation of [^{14}C]ergothioneine from the growth of *C. purpurea* on [2-^{14}C]acetate. The isolated ergothioneine was crystallized to constant activity. A final yield of 39·1 mg. of highly radioactive ergothioneine was obtained. This was diluted by adding to it ergothioneine hydrochloride (200 mg.). The material was dissolved in water and chromatographed on a column of Zeo-Karb 225, H^+ form, to convert the ergothioneine hydrochloride into the free base. The final yield of ergothioneine (free base) was 166·1 mg. (activity 1580 counts/min./mg. of C). This ergothioneine was used for the chemical degradation.

Degradation of [^{14}C]*ergothioneine isolated from the* [α-^{14}C]*histidine culture: oxidation of formic acid.* The ergothioneine (68·8 mg.) was degraded by the method described by Heath & Wildy (1956*b*), with the exception of the oxidation of formic acid obtained from the fission of the imidazole ring, which was carried out as follows. The aqueous solution remaining after ether extraction of the ethyl 4:5-dibenzamidopent-5-enoate, which contained sodium formate derived from the imidazole C-2 of ergothioneine, was heated to remove the dissolved ether. The remaining solution was transferred to a Quickfit flask, connected through a double-surface reflux condenser to a $Ba(OH)_2$ trap; 2N-H_2SO_4 (6 ml.) was added, the solution was boiled and a stream of N_2 was passed slowly through the apparatus until no more CO_2 was evolved. The formic acid was distilled from this residual solution by boiling in a CO_2-free apparatus; the formic acid and water distilled over together and in this way a separation from traces of residual ethyl dibenzamidopentenoate was achieved. The formic acid was oxidized in a CO_2-free apparatus by boiling for 1 hr. with 20 ml. of acid mercuric sulphate reagent (mercuric sulphate, 10 g.; CO_2-free water, 80 ml.; conc. H_2SO_4, 20 ml.). The CO_2 from the oxidation of formic acid was displaced by N_2 and collected as $BaCO_3$. This was filtered off and counted as a thin layer on the filter paper; yield of $BaCO_3$, 4 mg.

Investigation of the distribution of labelling in the amino acids of the protein of C. purpurea *grown in the presence of* [α-^{14}C]*histidine.* A small portion of the ethanol-insoluble 'protein' from *C. purpurea* was hydrolysed by heating in a sealed capillary in 6N-HCl at 100° for 16 hr. and then evaporated to dryness. A portion of the hydrolysate was chromatographed on a 10 in. square of Whatman no. 4 paper in phenol–aq. NH_3 soln. and butanol–acetic acid. A radioautograph was made of the resulting chromatogram.

Growth of C. purpurea *on a medium containing* [2(*ring*)-^{14}C]*histidine.* L-[2(ring)-^{14}C]Histidine (0·1 mc, 3·9 mg.) was added to 600 ml. of the mannitol–ammonium succinate medium, which was distributed between five 350 ml. Erlenmeyer flasks (120 ml. of medium/flask) and grown together with controls as described in the experiment with [α-^{14}C]histidine.

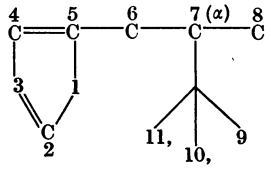
Ergothioneine was detected and estimated in the controls and ergothioneine hydrochloride (90 mg.) was added as carrier to the main growth; the labelled ergothioneine was then isolated by ion-exchange and alumina chromatography. The ergothioneine was crystallized to constant activity, yielding finally 33·3 mg. (activity 6840 counts/min./mg. of C). This ergothioneine was not degraded. L-Ergothioneine hydrochloride (204·3 mg.) was added to the mother liquors from the ergothioneine recrystallizations and the resulting solution was chromatographed on a column of Zeo-Karb 225 to convert the ergothioneine hydrochloride into the free base. The ergothioneine was crystallized to constant activity from aq. ethanol, yielding finally 97·5 mg. (activity 767 counts/min./mg. of C). This ergothioneine was used for degradation.

Degradation of [^{14}C]*ergothioneine isolated from the* [2(*ring*)-^{14}C]*histidine culture.* Ergothioneine (68·8 mg.) was degraded by the method described for the degradation of ergothioneine derived from [α-^{14}C]histidine. A portion of the ethanol-insoluble 'protein' from this culture was hydrolysed, the hydrolysate chromatographed and a radioautograph made of the chromatogram.

RESULTS

The distribution of radioactivity in the ergothioneine isolated from the [α-^{14}C]histidine culture and that isolated from the [2(ring)-^{14}C]histidine culture is shown in Table 1.

Table 1. *Distribution of radioactivity in ergothioneine isolated from cultures of* Claviceps purpurea *grown on media containing* [α-^{14}C]*histidine or* [2(*ring*)-^{14}C]*histidine*



Carbon atoms	[α-^{14}C]Histidine: Counts/min./mg. of C	[α-^{14}C]Histidine: Total counts/min.	[α-^{14}C]Histidine: Percentage of total	[2(ring)-^{14}C]Histidine: Counts/min./mg. of C	[2(ring)-^{14}C]Histidine: Total counts/min.	[2(ring)-^{14}C]Histidine: Percentage of total
2, 4, 5, 6, 7, 8, 9, 10, 11	1580	14 220	100·0	767	6900	100·0
9, 10, 11	0	0	0	27	81	1·2
4, 5, 6, 7, 8	2860	14 300	100·6	0	0	0
2	0	0	0	6554	6554	95·0

It can be seen that all the activity in the ergothioneine isolated from the [α-^{14}C]histidine culture was concentrated in the five-carbon chain (4, 5, 6, 7, 8). The carbon atoms of the betaine (9, 10, 11) and the imidazole C-2 were completely inactive. A radioautograph of a chromatogram of a protein hydrolysate from this [α-^{14}C]histidine culture showed that histidine had been partially degraded to glutamic acid. No activity was detected in the other amino acids.

Of the activity originally present in the [α-^{14}C]-histidine culture medium, 19% remained in the culture filtrate after growth, and of this 98·5% was adsorbed when the culture filtrate was applied to a Zeo-Karb column.

From Table 1 it can be seen that 95% of the activity in the ergothioneine isolated from the [2(ring)-^{14}C]histidine experiment was present in the imidazole ring C-2. A small activity was detected in the carbon atoms of the methyl groups of the betaine (9, 10, 11), whereas the five carbon atoms of the chain (4, 5, 6, 7, 8) were completely unlabelled.

The radioautograph of the chromatogram of hydrolysed protein revealed a very intense spot corresponding to histidine and three other weaker spots corresponding to methionine, serine and glycine.

Of the activity originally present in this [2-(ring)-^{14}C]histidine culture, 43% remained in the culture medium after growth. When this culture filtrate was applied to a column of Zeo-Karb 225, 39% of the activity was retained by the column.

DISCUSSION

The results of these experiments fully confirm that ergothioneine is formed by the fungus *Claviceps purpurea* directly from histidine without fission of the imidazole ring or alteration to the aliphatic side chain. The incorporation of [α-^{14}C]histidine into ergothioneine by *C. purpurea* clearly showed that histidine was a precursor, but it did not establish that the intact imidazole ring of histidine was directly utilized for the formation of ergothioneine. The possibility existed that ring fission of histidine could have occurred, followed by the reincorporation of a one-carbon compound linked through sulphur such as the $-SCH_3$ group of methionine. However, the ergothioneine isolated from the culture grown in the presence of ring-labelled histidine was highly labelled and the activity was concentrated in the imidazole C-2. The slight activity in the carbon atoms of the betaine showed that there had been some metabolism of the labelled histidine by the fungus, resulting in the liberation of labelled one-carbon fragments. This was confirmed by the radioautograph of the chromatogram of hydrolysed protein which showed the presence of radioactive methionine, serine and glycine. The high degree of incorporation of labelling into the imidazole C-2 of ergothioneine, however, showed that the intact imidazole ring of histidine had been incorporated into ergothioneine; had this labelling arisen by reincorporation from the one-carbon pool, the activity in this carbon atom would have been only of the order of that detected in the carbon atoms of the betaine.

Melville, Eich & Ludwig (1957) have recently studied the biosynthesis of ergothioneine by *Neurospora crassa* and they have shown on the basis of the high degree of incorporation of labelled histidine into the isolated ergothioneine that this organism also derived its ergothioneine by synthesis from histidine. As our results and those of Melville *et al.* (1957) are in complete agreement for two different fungi it would appear that the formation of ergothioneine from histidine is the normal pathway of this synthesis.

Some information on the metabolism of histidine by this fungus is obtained from the distribution of isotope in the culture broth after growth. All the amino acids, including histidine and glutamic acid, are retained by the ion-exchange resin, whereas the carboxylic acids, including formic acid, are not adsorbed. In the growth containing [2(ring)-^{14}C]-histidine, the liberation of one-carbon fragments not attached to nitrogen was demonstrated by the fact that 61% of the isotope was in the non-basic fraction, whereas in the experiment with [α-^{14}C]-histidine only 1·6% of the isotope was in this similar fraction. Radioautographs of two-dimensional chromatograms of the hydrolysed proteins from these experiments showed the presence of labelled methionine, serine and glycine derived from the ring C-2 but only glutamic acid derived from the α-carbon.

At least two steps are involved in the biological formation of ergothioneine from histidine: methylation of the amino nitrogen and attachment of sulphur to the C-2 of the imidazole ring. Thiolhistidine has never been shown to occur naturally and, moreover, synthetic L-2-thiolhistidine does not serve as a precursor of ergothioneine for *Neurospora crassa* (Melville *et al.* 1957). On the other hand, hercynin, the betaine of histidine, occurs naturally in certain fungi (Kutscher, 1910; Reuter, 1912) and would seem a likely intermediate in ergothioneine biosynthesis from histidine. If, in fact, this is so, methylation of histidine precedes sulphuration in the biosynthetic process.

SUMMARY

1. Cultures of *Claviceps purpurea* were grown on media containing either [α-^{14}C]histidine or

[2(ring)-^{14}C]histidine. Labelled ergothioneine was isolated from these cultures and chemically degraded.

2. The intact imidazole ring and side chain of histidine were shown to be incorporated into ergothioneine by this fungus.

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The Inhibition by γ-Irradiation of Incorporation of ^{32}P into Rat Thymocytes *in vitro*

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In a search for the primary biochemical lesions which are produced by ionizing radiation in animals, it would be convenient to have a mammalian system which is radiosensitive *in vitro* and which would provide sufficient material to permit a biochemical investigation. The inhibition of uptake of precursor into the nucleic acids of different tissues is a well-known consequence of irradiation *in vivo* (Euler & Hevesy, 1942) and can be detected very soon after the end of exposure (see Mitchell, 1956; Ord & Stocken, 1957). Howard & Pelc (1951), Lajtha, Oliver & Ellis (1954) and Totter (1954) have demonstrated the radiosensitivity of bean-root tips and bone-marrow cultures and suspensions to radiation *in vitro*, the inhibition of precursor uptake into nucleic acids being used as an index of radiation damage. Trowell (1952) found that cultures of lymph node are sensitive to exposure *in vitro*, and by using his cultures we have shown that incorporation of ^{32}P into their deoxyribonucleic acid is affected before histological damage can be detected (Ord & Stocken, 1956). On the other hand, the experiments on uptake of ^{32}P into the nucleic acids of thymus suspensions after exposure to 1000–2000 r. of γ-irradiation *in vitro* gave equivocal results (Ord & Stocken, 1956). The development of a medium suitable for the isolation of nuclei (Barnes, Esnouf & Stocken, 1957) prompted a re-examination of the behaviour of thymus suspensions in this medium, and they have now been found to be radiosensitive. It has therefore been possible to investigate some of the biochemical changes which accompany the inhibition of metabolism of thymus deoxyribonucleic acid.

METHODS

Animals. Young animals (70–130 g.) of the Laboratory stock of Wistar rats were used throughout. The results appeared to be independent of the sex and weight of the animal within this range.

Thymus suspensions. In the earlier experiments thymi from several rats were pressed through a stainless-steel gauze, suspended in a potassium-rich medium (0·085M-KCl, 0·0085M-NaCl, 0·0025M-$CaCl_2$, 0·0025M-$MgCl_2$, 0·005M-triethanolamine hydrochloric acid buffer, pH 7·2) supplemented with glucose (final concentration 10 mg./ml.) (Barnes *et al.* 1957) and filtered without squeezing through a single layer of muslin. This produced suspensions with not more than 30% of whole cells. To obtain 80–90% of undamaged cells, the capsule at the base of the thymus was sliced and the contents of the gland were gently scraped free. The operation was performed on a glass surface cooled with ice, and the resulting material was suspended in medium. From three to four rats 1 g. (wet wt.) of extruded thymocytes free from connective tissue was obtained, suspended in 4 ml. of medium and filtered as before. For irradiation and incorporation of ^{32}P, 1 ml. of suspension was incubated in Warburg flasks at 37°. It was thus possible to irradiate the cells while they were actively respiring and to measure the oxygen consumption during the experiment.

Cell counts. These were very kindly performed by Mr M. P. Esnouf by the differential-phase-contrast technique of Barer, Joseph & Esnouf (1956). Whole cells were distinguished from damaged and pycnotic cells and from nuclei. The results are given as total nuclei/ml. of suspension and as percentage of normal cells. Sufficient cells were counted to give an accuracy of ±5%.

Irradiation. Irradiation was provided by two 250 mc radium sources which were placed in Perspex holders beneath Warburg flasks (5 ml. capacity) containing 1 ml. of suspension (Ord & Stocken, 1955). With one source