sulphonated by reaction with sulphuric acid in the presence of catalyst.

3. The performance of the new reagent was tested in relation to the isolation of a large acidic peptide from the products of trypsin digestion of the 'B' chain of oxidized insulin.

The work described in this paper was carried out as part of the programme of the Food Investigation Board, of the Department of Scientific and Industrial Research. The commercial styrene and divinylbenzene were obtained through the courtesy of Dr T. R. E. Kressman of The Permutit Co. Ltd., London. One of us (J.F.) wishes to thank the Friends of the Hebrew University and the Humanitarian Trust for a maintenance grant.

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The Biosynthesis of Ergothioneine and Histidine by *Claviceps purpurea*

1. THE INCORPORATION OF [2-^{14}C]ACETATE

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Although ergothioneine occurs in blood (Hunter & Eagles, 1927), semen (Mann & Leone, 1953) and in various mammalian tissues, principally liver and brain (Melville, Horner & Lubschez, 1954), it would appear to be exogenous in origin, as no biosynthesis has been demonstrated in animals. Melville, Horner, Otken & Ludwig (1955) and Melville, Otken & Kovalenko (1955) studied the incorporation of ^{14}C-labelled histidine, formaldehyde, glycine and methionine by the rat and chicken and ^{35}S-labelled methionine and cystine by guinea pig, rat, pig and human, and in no case was there any demonstrable incorporation of isotope into the isolated ergothioneine. The earlier work of Heath, Rimington, Glover, Mann & Leone (1953) on the utilization of [^{35}S]methionine for this biosynthesis by the mature pig has not, after fuller investigation by these workers, been confirmed; the evidence now available is quite conclusive that, under the feeding conditions of the experiment, the seminal ergothioneine of the pig was not derived from the sulphur of methionine (Heath, Rimington & Mann, 1957).

In order to investigate the biosynthetic pathway of ergothioneine we decided to study the culture of ergot (*Claviceps purpurea*), from which ergothioneine was originally isolated by Tanret (1909). Much work on the artificial culture of this fungus has been carried out, principally to see whether the important pharmacological alkaloids could be formed. Nutritional requirements for submerged culture have been established by Tyler & Schwarting (1952). Although alkaloids are not formed in artificial culture by the standard strain of *C. purpurea*, Abe (1948) and Stoll, Brack, Kobel, Hofmann & Brunner (1954) have isolated two strains from *Agropyrum* and *Pennisetum* respectively, which produce some alkaloids (agroclavine and penniclavine) but not the principal naturally occurring alkaloids of ergot (ergotamine, etc.). This paper describes the isolation and degradation of ergothioneine from a culture of *C. purpurea* grown on a medium containing [2-^{14}C]acetate; a relationship between the biosynthesis of histidine and ergothioneine has been established. Ames & Mitchell (1955), Levy & Coon (1951, 1954), Ehrensvärd, Reio, Saluste & Stjernholm (1951) and Westley & Ciethaml (1956) have studied the biosynthesis of histidine by micro-organisms and it would appear that the five-carbon chain is not derived from glutamate or other derivatives of the citric acid

cycle. The present paper also reports the isolation and degradation of histidine from a culture of *C. purpurea* grown on a medium containing [2-^{14}C]-acetate.

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

EXPERIMENTAL

Organism. A culture of *Claviceps purpurea* (no. 44613) was obtained from the Commonwealth Mycological Institute, Kew.

[2-^{14}C]*Acetate.* This was obtained from the Radiochemical Centre, Amersham, with activities of 5 mc/m-mole (0·1 mc in 1·6 mg.) and 12 mc/m-mole (1·0 mc in 6·8 mg.).

Activated alumina. Type 'H', 100- to 200-mesh, was obtained from Peter Spence and Sons Ltd., Widnes.

Zeo-Karb 225. A 100- to 150-mesh, 4½% cross-linked resin was used, specially prepared by The Permutit Co. Ltd., London.

Oxoid membrane filters. These were obtained from Oxo Ltd., London.

Determination of radioactivity. All compounds were counted at infinite thickness in 1 cm.^{2} planchets with a thin mica end-window Geiger tube. Oxidation to CO_2 was carried out in the apparatus described by Popják (1955). The oxidant was prepared by dissolving CrO_3 (25 g.) in H_3PO_4 (sp.gr. 1·75) (167 ml.) and fuming H_2SO_4 (containing 20% of free SO_3) (333 ml.) and heating to 150° (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). Samples containing approx. 3 mg. of carbon were oxidized by heating with 4 ml. of oxidant in the evacuated apparatus, and the evolved CO_2 was collected as $BaCO_3$.

Sterilization. All media were sterilized by autoclaving at 10 lb./in.^{2} for 10 min.

Culture of organism. The standard strain was maintained and subcultured every 2 months on a medium consisting of: mannitol, 5·0 g.; Difco Bacto-Casitone, 0·5 g.; Difco yeast extract, 0·5 g.; KH_2PO_4, 0·1 g.; $MgSO_4,7H_2O$, 0·03 g.; agar, 1·5 g.; water to 100 ml.

A portion of the mycelium was transferred to a medium consisting of: mannitol, 5·0 g.; Difco yeast extract, 0·33 g.; ammonium succinate, 0·8 g.; $MgSO_4,7H_2O$, 0·03 g.; $ZnSO_4,7H_2O$, 0·0004 g.; $FeSO_4,7H_2O$, 0·0013 g.; KH_2PO_4, 0·1 g.; water to 100 ml. The inoculated medium was shaken in 350 ml. Erlenmeyer flasks (120 ml./flask) on a rotary shaker (80 rotations/min.) at room temp. (18–22°) for 10 days. The growth was filtered through a sterile cotton-wool plug to separate the mycelium, and 1 ml. of the filtrate, which consisted of a turbid suspension of conidia, was used to inoculate each 120 ml. of the following medium (used for the growth of the fungus for subsequent ergothioneine isolation): mannitol, 5·0 g.; ammonium succinate, 0·8 g.; $MgSO_4,7H_2O$, 0·03 g.; $ZnSO_4,7H_2O$, 0·0004 g.; $FeSO_4,7H_2O$, 0·0013 g.; KH_2PO_4, 0·1 g.; water to 100 ml. The growth conditions were the same as those described for the preparation of the inoculum.

Separation of conidia from mycelium. A culture of *C. purpurea* was filtered through a cotton-wool plug, which retained all the mycelium together with a small amount of conidia which could not be removed by washing. The conidia were collected by filtration through an Oxoid membrane, and washed and extracted for ergothioneine estimation.

Detection of ergothioneine. Ergothioneine can be readily detected in natural ergot sclerotia by the application of the Hunter diazo test (Hunter, Fushtey & Gee, 1949) to an aqueous extract. This is not possible with aqueous extracts of mycelium, owing to the presence of interfering substances which mask the typical diazo colour. The following procedure was therefore used.

The mycelium and conidia from a 10 days' shake culture (120 ml.) were harvested by filtration on an Oxoid membrane, washed three times with water, transferred to a 50 ml. centrifuge tube and extracted successively with 20, 10 and 10 ml. of hot water by immersing the tube in boiling water for 15 min. After centrifuging, the supernatants were evaporated to approx. 2 ml., ethanol (3 vol.) was added and, after centrifuging, the supernatant was taken to dryness. The residue was dissolved in water (0·3 ml.) and 30 μl. portions of this solution were subjected to descending chromatography on Whatman no. 4 paper with *n*-butanol–acetic acid (100:20), saturated with water. After drying in a neutral atmosphere, free from collidine and phenol, the chromatogram was exposed to NH_3 vapour, to neutralize any residual acetic acid, excess of NH_3 was removed and the chromatogram developed by dipping in 0·2% (w/v) ethanolic 2:6-dichloroquinonechloroimide (British Drug Houses Ltd.) and allowing to dry at room temp. The presence of ergothioneine is shown by the development of a brick-red colour which appears as the last traces of solvent evaporate from the chromatogram. The R_F of ergothioneine is 0·29. With this developing agent, other aromatic compounds develop grey colours on further standing.

Estimation of ergothioneine. The ethanol-treated aqueous extract of the fungus, obtained as described previously, was evaporated to dryness and dissolved in the minimum volume of an ethanol–water–formic acid mixture (75:25:1, by vol.). The ethanol was previously purified by distillation from Mg and I_2 and redistillation from Zn. This extract was applied to a 20 cm. × 0·9 cm. column of activated alumina previously washed and packed with the same aqueous ethanol–formic acid mixture (Melville *et al.* 1954). The column was developed with this solvent, 4 ml. fractions being collected. The fractions were evaporated to dryness and the ergothioneine in each estimated by the Hunter diazo reaction (Hunter, 1949). Colour intensities were measured on a Unicam spectrophotometer model SP. 350 at 525 mμ. Ergothioneine is eluted in fractions representing 35–50 ml. of solvent flow.

Isolation of [^{14}C]*ergothioneine*

Three separate experiments consisted of: (*a*) forty-eight flasks, each containing medium supplemented with 1 μc of [2-^{14}C]acetate/flask; (*b*) one flask with medium containing 52 μc of [2-^{14}C]acetate; (*c*) five flasks each with medium containing 200 μc of [2-^{14}C]acetate/flask. Control unlabelled cultures were simultaneously grown and their ergothioneine contents measured. The method of isolation in all three experiments was the same in principle, differing only in sizes of columns used. Experiment (*a*) was carried out to determine the degree of incorporation of isotope into ergothioneine. Experiment (*b*) did not yield ergothioneine of sufficient activity for complete degradation. Experiment (*c*) is described in detail.

After 10 days' growth, the fungus was collected on an Oxoid membrane and washed 3 times with water; a portion of the filtrate and washings was used to determine the

residual activity in the culture medium. L-Ergothioneine hydrochloride (90 mg.) was added to the moist mycelium, which was extracted as described above with a total of 200 ml. of water. The residual mycelium was boiled with 100 ml. of water and filtered. The aqueous extracts were combined and concentrated under reduced pressure to 50 ml., and ethanol (150 ml.) was added. After standing at −10° overnight, the 'protein' was removed by centrifuging, washed twice with 10 ml. of 75% ethanol and the combined supernatants were taken to dryness.

The residue was dissolved in 100 ml. of water and applied to a 10 cm. × 0·9 cm. column of Zeo-Karb 225 (H^+ form). Ergothioneine and other cations were retained. The column was washed with 50 ml. of water and then eluted with 0·1 N-NH_3 soln.; 3 ml. fractions were collected. Each fraction was spot-tested on filter paper with dichloroquinonechloroimide for the presence of ergothioneine. Ergothioneine is eluted just before the amino acids and all fractions containing ergothioneine were combined and evaporated to dryness.

The dried residue was dissolved in the minimum amount of aqueous ethanol–formic acid mixture and chromatographed, as described, on a 20 cm. × 0·9 cm. column of activated alumina. Fractions (4 ml.) were collected and spot-tested on paper with dichloroquinonechloroimide. The fractions were also spot-tested with ninhydrin. All fractions containing ergothioneine were combined, evaporated to dryness, dissolved in water and applied to a 10 cm. × 0·9 cm. column of Zeo-Karb 225 (H^+ form) to remove traces of Al^{3+} ions. The column was washed with water, which removed residual formic acid, and eluted with 0·1 N-NH_3 soln.; 4 ml. fractions were collected and spot-tested for ergothioneine and amino acids. All fractions containing ergothioneine were combined and evaporated to dryness.

Normally this procedure results in the complete separation of ergothioneine from all ninhydrin-reacting materials, but in this particular case a yellow ninhydrin test was given which was due to proline. The residue was therefore rechromatographed as described above, through two alumina columns, 20 cm. × 0·9 cm., and another 5 cm. × 0·9 cm. Zeo-Karb column. The ergothioneine thus obtained was free from any ninhydrin-reacting material. The dried residue was extracted 3 times with ethanol. The white insoluble residue of ergothioneine was dissolved in water, filtered, concentrated and crystallized from aqueous ethanol. It was counted at infinite thickness in 1 cm.^{2} planchets and recrystallized 3 times to constant specific activity. A final yield of 41 mg. of ergothioneine (free base) was obtained. (Activity 497 counts/min./mg. of carbon.)

Degradation of ergothioneine

Oxidation of ergothioneine. A sample (5 mg.) of ergothioneine was totally oxidized. The $BaCO_3$ obtained from the resulting CO_2 was filtered off, dried, weighed and plated in 1 cm.^{2} polythene planchets, from a suspension in 80% ethanol, and carefully dried under an infrared lamp.

Removal of trimethylamine. Ergothioneine (I; 29·4 mg.) was heated in a boiling-water bath with 60% KOH (1·0 ml.) for 3 hr. in a flask connected through a condenser to 0·3 N-HCl (10 ml.). Steam was passed through the flask until 50 ml. of distillate had been collected. The solution of trimethylamine hydrochloride thus obtained was evaporated to dryness, oxidized to CO_2 and counted as $BaCO_3$.

Reduction of 2-thiolurocanic acid. The residual yellow KOH solution of 2-thiolurocanic acid (II) was reduced with 2·5% sodium amalgam (2 g.) to yield β-(2-mercaptoglyoxalin-5-yl)propionic acid [2-thiolimidazole-4β-propionic acid (III)]. The resulting colourless solution was separated from Hg and passed through a 15 cm. × 0·9 cm. column of Zeo-Karb 225, (H^+ form) which retained the K^+ and Na^+ ions; the effluent contained, besides thiolimidazolepropionic acid, traces of silicic acid dissolved from the flask by the hot KOH. This

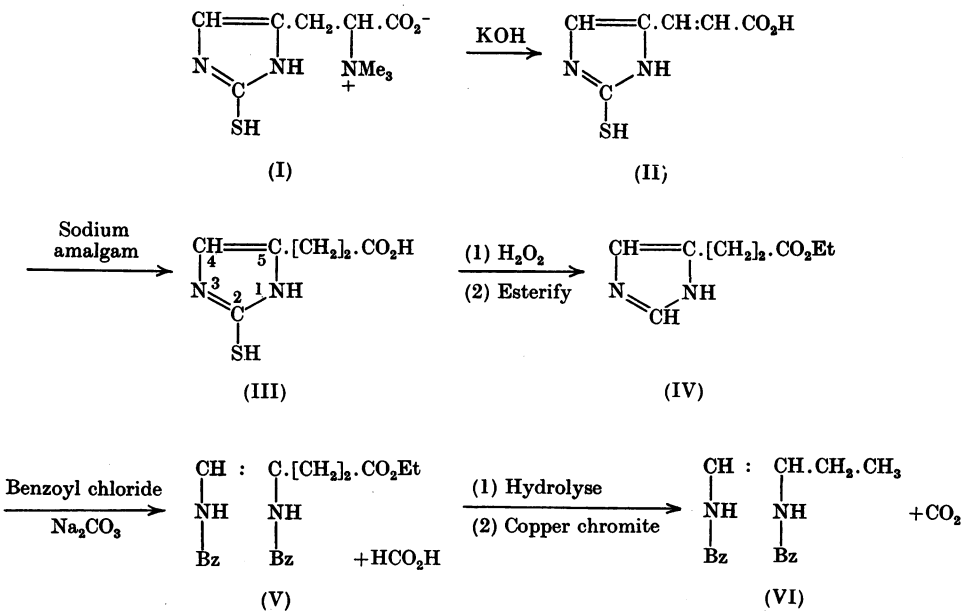


Fig. 1. Degradation of ergothioneine.

was removed by evaporating the solution to dryness and extracting the thiolimidazolepropionic acid with ethanol. A portion (5 mg.) of the isolated thiolimidazolepropionic acid was converted into $BaCO_3$. These reactions were followed by one-dimensional butanol–acetic acid chromatograms developed with dichloroquinonechloroimide, to ensure that all reactions had been taken to completion.

Desulphuration of 2-thiolimidazole-4β-propionic acid. The remainder of the thiolimidazolepropionic acid (16 mg.) was dissolved in water (5 ml.), treated with '30 volume' H_2O_2 (0·25 ml.) and heated for 5 min. on a boiling-water bath; the solution was spot-tested with dichloroquinonechloroimide for absence of positive reaction (thiolimidazoles give a red colour with this reagent and the corresponding imidazoles resulting from desulphuration give no immediate reaction). One-dimensional chromatograms, with butanol–acetic acid, developed with the Pauly diazo reagent, showed a single spot corresponding to imidazole-4β-propionic acid.

The solution was neutralized with 0·1 N-NaOH (1·6 ml.) and evaporated to dryness. The dried residue was refluxed with saturated ethanolic HCl (20 ml.) for 1 hr., benzene (5 ml.) was added, the solvent was distilled off and the residue was dried over NaOH in a vacuum desiccator.

Benzoylation of ethyl imidazole-4β-propionate. The ethyl imidazolepropionate (IV) was dissolved in water (5 ml.); Na_2CO_3 (0·2 g.) was added and, after cooling to 0°, benzoyl chloride (42 mg.) was added. The mixture was shaken at 0° for 1 hr. and then extracted with ether. After removal of the ether, the resulting ethyl 4:5-dibenzamidopent-5-enoate (V) was crystallized 3 times from aqueous ethanol in fine needles. A portion (3·6 mg.) was converted into $BaCO_3$. The ether-extracted sodium carbonate solution, which contained sodium formate derived from the imidazole C-2 of ergothioneine, was concentrated to about 2 ml. in a 25 ml. flask; a reflux condenser leading into a $Ba(OH)_2$ trap was then fitted. The solution was acidified with dil. H_2SO_4 and a stream of N_2 was passed through the boiling liquid until no more CO_2 (derived from the Na_2CO_3) was evolved. The residual formic acid was then oxidized with $KMnO_4$ and the CO_2 collected as $BaCO_3$.

The remaining ethyl 4:5-dibenzamidopent-5-enoate (1·8 mg.) was diluted with unlabelled ethyl 4:5-dibenzamidopent-5-enoate (18 mg.) and dissolved in ethanol (1 ml.). To this 10% ethanolic KOH (0·5 ml.) was added and the ester hydrolysed by heating under reflux on a water bath for 10 min. After neutralizing with HCl the free acid was crystallized from aqueous ethanol as tufts of fine hairs, m.p. 150–152° (Windaus, Dörries & Jensen, 1921).

4:6-Dibenzamidopent-5-enoic acid (13·9 mg.) was mixed with copper chromite catalyst (0·5 g.) (Lazier & Arnold, 1939) in the oxidation apparatus. After evacuation, the flask was heated to 275° in a bath of Wood's metal. The CO_2 derived from the carboxyl carbon was trapped as $BaCO_3$.

Preparation of 3:4-*dichlorobenzenesulphonic acid*

The 3:4-dichlorobenzenesulphonic acid used for the isolation of histidine was prepared by boiling 25 g. of 3:4-dichlorobenzenesulphonyl chloride (British Drug Houses Ltd.) in water (200 ml.) for 12 hr. The colourless aqueous solution was separated from a small amount of brown oil and concentrated to a small volume under reduced pressure. Chloroform (25 ml.) was added and, after standing, the crystals of 3:4-dichlorobenzenesulphonic acid were collected and dried in a vacuum desiccator over NaOH. Wt., 25·4 g.; m.p. 54°.

Isolation of [^{14}C]*histidine*

The washed ethanol-insoluble 'protein' (0·5 g.) was hydrolysed by refluxing in 6 N-HCl (100 ml.) for 20 hr., distilled to dryness and dried in a vacuum desiccator over NaOH. The residue was dissolved in water, filtered and adjusted to pH 4 with 2 N-NaOH. L-Histidine hydrochloride monohydrate (225 mg.) was added as carrier, followed by saturated ethanolic $HgCl_2$ (10 ml.). The pH was adjusted to 7·2 with saturated $NaHCO_3$ soln. to precipitate the Hg 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 5 ml. and 3:4-dichlorobenzenesulphonic acid (1 g.) was added to the hot solution. After standing overnight at 4°, the crystals of L-histidine bis-3:4-dichlorobenzenesulphonate (Vickery, 1942) were filtered off, and washed with ice-cold water; wt., 561 mg. The radioactivity was determined at infinite thickness after packing a finely ground suspension in acetone in 1 cm.^{2} planchets. The histidine bis-3:4-dichlorobenzenesulphonate was recrystallized 4 times from hot water, to yield 312 mg. of constant activity (288 counts/min./mg. of carbon).

Degradation of [^{14}C]*histidine*

Decarboxylation. [^{14}C]Histidine bisdichlorobenzenesulphonate (100 mg.) and ninhydrin (250 mg.) in water (5 ml.) were boiled for 8 min. in the oxidation apparatus. The CO_2 evolved was collected as $BaCO_3$.

Benzoylation. [^{14}C]Histidine bisdichlorobenzenesulphonate (110 mg.) was refluxed for 30 min. with saturated methanolic HCl; the solvent was removed by vacuum distillation and the residue dried over NaOH in a vacuum desiccator. The methyl ester was dissolved in water (10 ml.), cooled to 0° and Na_2CO_3 (0·4 g.) was added, followed by benzoyl chloride (80 mg.). The mixture was shaken for 1·5 hr. at 0°. At the end of this time the precipitate of methyl 2:4:5-tribenzamidopent-5-enoate was filtered; some adhered to the walls of the flask, owing to the presence of unreacted benzoyl chloride; this, together with the filtered precipitate, was dissolved in ethanol and heated to decompose the benzoyl chloride. The solution was concentrated and crystallized by the addition of water. After standing at 4° overnight the crystals were filtered; wt., 17·2 mg. This was recrystallized to constant activity from

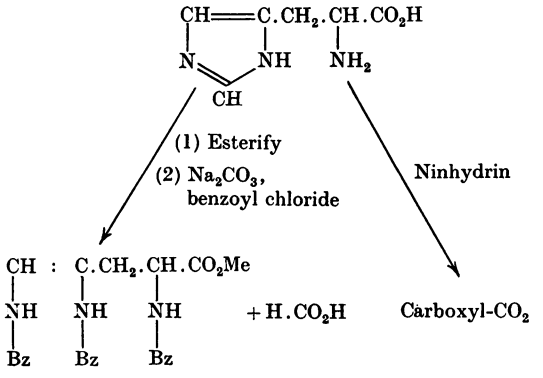


Fig. 2. Degradation of histidine.

aqueous ethanol. For radioactivity measurement this compound was packed from a suspension in 70% (v/v) ethanol and dried under an infrared lamp.

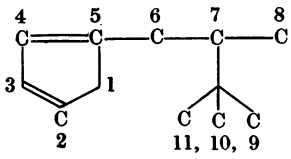
An attempt to collect the formate carbon as $BaCO_3$ by a method analogous to that used for the ergothioneine 'formate' was not successful owing to the oxidation of the dichlorobenzenesulphonic acid by the permanganate.

RESULTS

The distribution of radioactivity in the isolated ergothioneine is shown in Table 1, that in the isolated histidine in Table 3. All compounds were counted with an end-window Geiger–Müller counter (as $BaCO_3$ at infinite thickness) and the results are expressed as counts/min./mg. of carbon. All samples were counted 3 times for not less than a total of 2500 counts and checked against a standard source.

It can be seen from Table 1 that the highest labelling in the ergothioneine is in the ring C-2 with 2411 counts/min./mg. of carbon, followed by the methyl carbons of the betaine (9, 10, 11) with 500 counts/min./mg. of carbon. The remaining five-carbon residue (4, 5, 6, 7, 8) contained only 113 counts/min./mg. of carbon, and of this the carboxyl carbon (8) gave 216 counts/min./mg. of carbon. The low activity of the five-carbon chain, and in particular of carbon atoms 5, 6 and 7, showed that glutamate derived from acetate via the citric acid cycle was not the precursor of this chain. The general distribution of radioactivity in the amino acids formed by *C. purpurea* grown on a medium containing [2-^{14}C]acetate was studied in order to ascertain that the general pattern of labelling was what one would expect if the citric acid cycle were operating. No attempt was made to isolate each individual amino acid, but to obtain a qualitative picture of the isotope distribution an acid hydrolysate of the alcohol-insoluble protein obtained during the isolation of ergothioneine was subjected to two-dimensional chromatography in phenol–ammonia and butanol–acetic acid. After development with ninhydrin and identification, a circle of 1 cm.^{2} area was removed from the centre of those amino acid spots which were clearly separated and counted with the Geiger-Müller end-window tube. The results are given in Table 2. These results are not strictly comparable, partly because of the loss of the carboxyl carbon during ninhydrin development and partly because of the different concentrations of amino acid in each spot, even though circles were removed only from spots of comparable intensities. The loss of carboxyl carbon by ninhydrin treatment was measured on a portion of the hydrolysate. Untreated, this gave 1705 counts/min.; after ninhydrin treatment this gave 1630 counts/min., i.e. a loss of 4·4%. Nevertheless, the results obtained were, in general, similar to those of Bloch (1951) for the distribution of isotope in yeast protein derived from [2-^{14}C]acetate. It will be noted that the activities, as measured by this method, are low, owing to the absorption of the radiation by the paper and to the very small quantities present in the surface of each paper circle. A sample of proline which was isolated had an activity greater than 10 000 counts/min./mg. of carbon; this, coupled with the fact that the glutamic acid spot on the chromatogram was the most active, is evidence that the five-carbon chain in ergothioneine was not derived from glutamate. This had been shown to be the case also for the five-carbon chain in histidine by Levy & Coon (1954) and it seems, therefore, that there may be similarities between the pathways of histidine and ergothioneine biosynthesis. For this reason we isolated and degraded the histidine formed by the *C. purpurea* grown on the medium containing [2-^{14}C]-acetate. These results are given in Table 3. It will be noted that the highest activity, as in the ergo-

Table 1. *Distribution of radioactivity in ergothioneine isolated from a culture of* Claviceps purpurea *grown on a medium containing* [2-^{14}C] *acetate*



Carbon atoms	Counts/min./mg. of C	Total counts/min.	Percentage of total
2, 4, 5, 6, 7, 8, 9, 10, 11	497	4476	100·0
2, 4, 5, 6, 7, 8	496	2976	66·5
9, 10, 11	500	1500	33·5
4, 5, 6, 7, 8	113	565	12·6
2	2411	2411	53·9
8	216	216	4·8
* 4, 5, 6, 7	87	348	7·8

* Obtained by difference.

Table 2. *Radioactivity of* 1 *cm.*^{2} *circles taken from a ninhydrin-developed, two-dimensional chromatogram of the hydrolysate of protein obtained from a culture of* Claviceps purpurea *grown on a medium containing* [2-^{14}C]*acetate*

Amino acid	Counts/min.
Glutamic acid	79
Lysine	42
Leucine	34
Proline	31
Aspartic acid	29
Threonine	11
Tyrosine	7
Alanine	5
Glycine	2
Phenylalanine	2

thioneine, is in the C-2 of the imidazole ring (1368 counts/min./mg. of carbon), with an average of 72 counts/min./mg. of carbon in the five-carbon chain, and of this the carboxyl carbon contained 142 counts/min./mg. of carbon, leaving a low activity in carbon atoms 5, 6 and 7, which would have been the most active if this chain had been derived from acetate via glutamate (Ehrensvärd *et al.* 1951).

In Table 4 a comparison is made between the distribution of activity in histidine and the corresponding six-carbon compound, imidazolepropionic acid, derived from ergothioneine. It can be seen that the distribution of isotope is almost identical.

Observations on the chemical degradation

Fig. 1 shows the method of chemical degradation of ergothioneine. All reactions were first studied with unlabelled material and adapted for the micro quantities involved.

Complete removal of trimethylamine from ergothioneine by the action of alkali is not easily achieved. The end products of this reaction were studied by one-dimensional paper chromatography with butanol–acetic acid and by developing with dichloroquinonechloroimide (ergothioneine, R_F 0·29; 2-thiolurocanic acid, R_F 0·71; 2-thiolimidazolepropionic acid, R_F 0·66). Although the R_F values of thiolurocanic acid and thiolimidazolepropionic acid are not greatly different in either this or other solvents tried, the colour given by the developing agent is brick-red with ergothioneine and thiolimidazolepropionic acid and purple with thiolurocanic acid. It was found that some ergothioneine still remained after heating for 1 hr. at 100° in 10% sodium hydroxide or standing at room temperature for 48 hr. in 40% sodium hydroxide. Treatment of ergothioneine in 60% potassium hydroxide at 100° for 3 hr. resulted in the complete conversion of ergothioneine into thiolurocanic acid.

The removal of the potassium hydroxide after the completion of this reaction was originally performed by electrolytic desalting. This caused the simultaneous reduction of thiolurocanic acid to thiolimidazolepropionic acid. The method was abandoned, however, in favour of reduction with sodium amalgam and decationization with Zeo-Karb 225, as the latter proved simpler and safer for the micro quantities involved.

In order to study subsequent stages in the degradation, 20 g. of thiolimidazolepropionic acid was synthesized by the method of Heath, Lawson & Rimington (1951) for 4-methyl-2-thiolimidazole but starting from glutamic acid instead of alanine.

Desulphuration of thiolimidazolepropionic acid with Raney nickel was not as suitable as controlled oxidation with peroxide, owing to the formation of a nickel co-ordination complex with the resulting imidazolepropionic acid; this made isolation difficult.

Decarboxylation of 4:5-dibenzamidopent-5-enoic acid was achieved by heating in a Wood's metal bath to 275° in preference to heating in quinoline, owing to the high blank obtained by the latter method.

Observations on the culture of Claviceps purpurea

This strain of *C. purpurea* has been used for a year and it has been observed that the quantity of ergothioneine produced is increasing. This is shown in Table 5. Ergothioneine is not present in the culture broth and is confined to the conidia. A typical 10 days' shake culture in 120 ml. of medium

Table 3. *Distribution of radioactivity in histidine isolated from a culture of* Claviceps purpurea *grown on a medium containing* [2-^{14}C]*acetate*

Carbon atoms	Counts/min./mg. of C	Total counts/min.	Percentage of total
2, 4, 5, 6, 7, 8	288	1728	100·0
4, 5, 6, 7, 8	72	360	20·9
*2	1368	1368	79·1
8	142	142	8·2
* 4, 5, 6, 7	54	216	12·5

* Obtained by difference.

Table 4. *Comparison of the distribution of radioactivity in histidine and in imidazolepropionic acid derived from ergothioneine isolated from the same culture of* Claviceps purpurea *grown on a medium containing* [2-^{14}C]*acetate*

Carbon atoms	Radioactivity as percentage of total in: Histidine	Imidazolepropionic acid
2, 4, 5, 6, 7, 8	100·0	100·0
2	79·1	81·0
4, 5, 6, 7, 8	20·9	19·0
4, 5, 6, 7	12·7	11·7
8	8·2	7·3

Table 5. *Ergothioneine formed in standard* 120 *ml. cultures of* Claviceps purpurea, *demonstrating the increase in the ability of the fungus to produce ergothioneine with repeated subculturing*

Date	Total ergothioneine (μg.)/120 ml., 10 days' culture
21. ix. 55	260
3. xi. 55	296
15. ii. 56	451
2. iii. 56	655
2. iii. 56*	152

* This was a culture of *Claviceps purpurea* as obtained from the Commonwealth Mycological Institute, Kew, which had not been subcultured on our medium.

was shown to contain 615 μg. of ergothioneine in the conidia, no ergothioneine in the culture broth and 40 μg. of ergothioneine in the mycelium, probably due to contamination with conidia. The mannitol concentration of the medium determined polarimetrically as the sodium borate complex (Raistrick & Young, 1931) had fallen from 5 to 1·3 % and free ammonia was no longer present.

Dr A. Stoll of Sandoz Ltd., Basle, kindly sent us a culture of a strain of *C. purpurea* which produces some ergot-type alkaloids in artificial culture. This culture was examined for both alkaloid and ergothioneine production. It was found that after 10 days' growth, under our usual conditions, alkaloids had not been formed but the total growth contained 226 μg. of ergothioneine/flask. Alkaloids were present after 6 weeks' growth. Stoll *et al.* (1954) have shown that there is little, if any, alkaloid production in the first 20 days, and he normally cultures for over 30 days. It is apparent that ergothioneine is synthesized before the alkaloids and that there does not appear to be a relationship between ergothioneine and alkaloid synthesis.

To study the effect of possible ergothioneine precursors, which were not available as labelled compounds, their effect on the quantity of ergothioneine produced when they were added to the usual medium was observed. The results are shown in Table 6.

All cultures were treated identically with regard to medium, inoculum, temperature and sterilization, except that the glucosamine was sterilized by filtration and then added to the autoclaved control medium. It will be seen that the addition of histidine did not significantly alter the amount of ergothioneine formed, whereas the presence of ergothioneine in the culture medium resulted in greater accumulation of ergothioneine within the total growth. The presence of the small quantity of glucosamine not only lowered the amount of ergothioneine formed but also altered the fungus morphologically; it was less pigmented and the growth consisted of small spheres of mycelium with fewer conidia than the usual culture, which normally consists of a thick mass of mycelial threads containing a high percentage of conidia.

Table 6. *Effect of additions to the standard medium on the production of ergothioneine in a* 10 *days'* (120 *ml.*) *shake culture of* Claviceps purpurea

Additions	Total ergothioneine (μg.) in mycelium and conidia
None	263
None	280
None	308
Histidine (10 mg.)	313
Histidine (10 mg.)	310
Glucosamine HCl (10 mg.)	170
Glucosamine HCl (10 mg.)	205
Ergothioneine (1 mg.)	435
Ergothioneine (1 mg.)	386

DISCUSSION

The results presented above show that when *C. purpurea* is grown on a chemically defined medium, ergothioneine is produced. This is the first report of the presence of ergothioneine in a micro-organism grown on a chemically defined medium. Ergothioneine was originally isolated from the sclerotia of ergot (Tanret, 1909) which also contain the important pharmacological ergot alkaloids. It has, however, been shown by Abe (1948) and Stoll *et al.* (1954) that certain alkaloids can be produced in artificial cultures where there is no sclerotia formation. It is now clear that sclerotia formation is not necessary for ergothioneine production. Our results have shown that the ergothioneine formed in artificial culture is confined principally to the conidia; the small amount detected in the separated mycelium was attributed to conidial contamination. No ergothioneine was present in the culture broth. This is not in agreement with the work of Garay (1956), who studied the distribution of ergothioneine between conidia and honey-dew formed during the parasitic growth of *C. purpurea* on rye. Under these conditions Garay found that the conidia did not contain ergothioneine but that this was present in the conidia-free honey-dew. Although the 10 days' shake cultures of *C. purpurea* contained ergothioneine they did not contain alkaloids and therefore ergothioneine production is not related to alkaloid formation. This was further confirmed by our work on the alkaloid-producing strain of this fungus which was kindly provided by Dr A. Stoll. This was shown to contain ergothioneine after 10 days' growth, at which time alkaloid formation had not yet occurred, although alkaloids were subsequently formed.

The fact that ergothioneine was present in the conidia, together with the reported occurrence of ergothioneine in plant seeds such as oats (Melville & Eich, 1956) and the known occurrence of ergothioneine in semen (Mann & Leone, 1953), suggested to us that a possible function of ergothioneine was connected with germination. However, in preliminary experiments no difference in the rate of growth could be detected when cress seeds were germinated either in the presence or absence of ergothioneine.

The results of the degradation of the [^{14}C]ergothioneine show that the major contribution of the methyl group of acetate to the ergothioneine molecule is in the provision of one-carbon moieties to provide the C-2 of the ring and the methyl groups of the betaine. Similar results were obtained for the

degradation of the isolated histidine. It can be concluded from the almost identical percentage distribution of isotope in both ergothioneine and histidine, as shown in Table 3, that the pathways of synthesis of these two compounds are identical. Owing to the numerous pathways of utilization of acetate the actual proportion of the acetate C-2 incorporated into the histidine and ergothioneine would have been small and was not determined. The results obtained are based on the analysis of representative samples of ergothioneine and histidine, and the conclusions are based on the ratio of labelling between the atoms of the molecules and not the absolute amount of isotope incorporated.

Levy & Coon (1954) showed that the methyl groups of acetate can serve as a source of 'formate', which they had previously shown (Levy & Coon, 1951) to be a precursor of the C-2 of the imidazole ring of histidine isolated from yeast. They found that neither [1-^{14}C]glycine nor $^{14}CO_2$ was incorporated into the histidine, and from this they concluded that the formate is not oxidized to carbon dioxide before incorporation and, moreover, that the imidazole ring of histidine is not derived from purines. However, they did not detect any incorporation of the methyl group of acetate into the five-carbon chain of histidine, possibly because of the low degree of labelling with which they were working.

Ehrensvärd *et al.* (1951) showed that the methyl group of acetate was the precursor of carbon atoms 2, 3 and 4 of glutamic acid isolated from yeast grown on acetate as the sole source of carbon. From our results it is apparent that, owing to the low labelling in carbon atoms 5, 6 and 7 of the isolated ergothioneine, the five-carbon chain could not have been derived from glutamate. The non-participation of the citric acid cycle in the formation of the five-carbon chain in histidine can be deduced from the work of Ames & Mitchell (1955), who showed that mutants of *Neurospora crassa* that require histidine accumulated the phosphates of imidazole–glycerol, imidazole–acetol and histidinol, which suggested that a pentose was the precursor of this chain. This mode of formation has been further elucidated by Westley & Ciethaml (1956), who, working with mutants of *Escherichia coli* that require histidine, and specifically labelled glucose, obtained a pattern of labelling in their isolated histidinol which showed that the five-carbon chain was not derived directly from a pentose formed via the glucose monophosphate shunt, but was formed from the condensation of two- and three-carbon fragments resulting from glycolysis of the labelled glucose. Ehrensvärd *et al.* (1951) have also shown that the carboxyl carbon of histidine isolated from yeast is derived from the methyl group of acetate.

Evidence that acetate is being incorporated into amino acids via the citric acid cycle is presented in Table 4. The relative amounts of labelling in these amino acids are roughly similar to those obtained by Bloch (1951), who studied the incorporation of labelled acetate into yeast protein. The highest labelling, which is in the glutamate, is consistent with the direct condensation of labelled acetate with oxaloacetate to yield highly active α-oxoglutarate and hence glutamate. The activity of aspartate derived from oxaloacetate will be further diluted because of the added succinate of the medium, and alanine derived from pyruvate will be still further diluted because of inactive pyruvate derived from the carbohydrate of the medium.

Thiolhistidine, the amino acid corresponding to ergothioneine, could be a possible precursor of ergothioneine in this fungal system, although it has been shown that, in rats, thiolhistidine is metabolically inactive (Heath, 1953). During our chromatographic work we failed to detect its presence. As this compound separates readily from ergothioneine in the solvent systems used and develops the characteristic brick-red colour with dichloroquinonechloroimide we would undoubtedly have detected its presence had it been formed in the fungus; we therefore consider that it is unlikely that free thiolhistidine is a precursor of ergothioneine. The possibility that formation of the thiolimidazole moiety is by the direct addition of a sulphur compound to the imidazole ring of histidine is being further studied.

Although the results of our isotope experiments show a relationship between histidine and ergothioneine, the addition of small quantities of histidine to the culture medium had no effect on the quantity of ergothioneine produced. Glucosamine was added because it appears from the work of Ames & Mitchell (1955) that the side chain of histidine at an early stage in its genesis is hydroxylated, and the amino aldehyde group in glucosamine would yield a chemically reactive site for the condensation of thiocyanate to yield a hydroxylated thiolimidazole, with a four-carbon side chain. The inhibition of ergothioneine synthesis by addition of a small quantity of glucosamine (0·007 %) might be due to competitive interference with a similar condensation between thiocyanate and a pentosamine.

Two observations incidental to our work are of interest. The characteristics of our culture of *C. purpurea* changed during the course of the experiments: a progressive increase in ergothioneine synthesis took place and at the same time the conidia became progressively redder.

At one stage in the work *C. purpurea* was acetone-dried and it was found that most of the ergothioneine was extracted into the acetone. This did not take place if the mycelium was dried before extraction.

SUMMARY

1. The growth of *Claviceps purpurea* in liquid shake culture, and methods for the detection, estimation and isolation of ergothioneine from these cultures are described.

2. Methods for the degradation of ergothioneine on a micro scale are given.

3. The isolation of histidine from a protein hydrolysate, and its degradation, are described.

4. The results obtained from the degradation of ergothioneine and histidine isolated from a culture of *Claviceps purpurea* grown on a medium containing [2-^{14}C]acetate show that the pathways of biosynthesis of these two compounds are identical in this fungus.

5. The major contribution of acetate to the ergothioneine molecule is in the provision of one-carbon moieties, and the highest labelling was in the carbon-2 of the imidazole ring, followed by the methyl groups of the betaine. The residual five-carbon chain was labelled to a lesser extent. The distribution of isotope in this chain precludes the possibility of it having been derived from glutamate formed via the citric acid cycle. Evidence was obtained that acetate was incorporated into other amino acids via the citric acid cycle.

6. Continual subculturing has produced a strain of *Claviceps purpurea* with pigmented conidia and in which the yield of ergothioneine is steadily increasing.

7. Ergothioneine production by *C. purpurea* is independent of both alkaloid and sclerotia formation. The ergothioneine was found wholly or principally in the conidia.

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