Biosynthesis of Ergotamine by *Claviceps purpurea* (Fr.) Tul.

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High-yielding strains of *Claviceps purpurea* (Fr.) Tul, grown on a defined medium, have been used for a study of the biosynthesis of the peptide ergot alkaloid, ergotamine. L-[U-¹⁴C]tryptophan, DL-[2-¹⁴C]mevalonic acid lactone, sodium [2-¹⁴C]acetate, sodium [¹⁴C]formate and the methyl group of L-[methyl-¹⁴C]methionine were efficiently incorporated into the peptide alkaloids and specifically labelled the ergoline moiety of ergotamine. These results are the same as previously found for the biosynthesis of other ergot alkaloids. Time-course incubation experiments demonstrated that L-[U-¹⁴C]phenylalanine, L-[U-¹⁴C]proline and L-[U-¹⁴C]alanine were incorporated into the peptide ergot alkaloids. Chemical degradation of the radioactive alkaloid derived from additional precursor incubation experiments showed that phenylalanine and proline function as the most efficient precursors, and specifically label the constitutive side-chain phenylalanyl and prolyl moieties of the alkaloid. The evidence obtained from L-[U-¹⁴C]alanine-incorporation experiments was inconclusive. However, degradation of ergotamine isolated after incubation with DL-[1-¹⁴C]alanine, showed that the carboxyl group of the labelled amino acid was specifically incorporated into the α -hydroxy- α -amino acid residue of the alkaloid. This, in conjunction with the L-[U-¹⁴C]alanine-incorporation results, showed conclusively that all three carbon atoms of alanine were incorporated as a biosynthetic unit into the α -hydroxy- α -amino acid moiety of ergotamine.

In the field of ergot-alkaloid biosynthesis (I shows the structure of ergotamine) most investigations have been concerned with the origin of the ergoline nucleus of the simple clavine alkaloids (for reviews see Agurell, 1966; Ramstad, 1968). However, it has been demonstrated that the same ergoline precursors, namely tryptophan and indole (Paul, 1957; Baxter *et al.*, 1960, 1961; Taber & Vining, 1959; Vining & Taber, 1963; Gröger & Erge, 1970), mevalonic acid (Baxter

et al., 1961; Taylor & Ramstad, 1960) and the methyl group of L-methionine (Majer *et al.*, 1967; Castagnoli *et al.*, 1970), function as specific precursors for the lysergic acid moiety of the peptide alkaloids.

Mainly because of the lack of organisms suitable for the production of these alkaloids in sufficient yields in submerged culture, the biosynthesis of the side chain of the peptide alkaloids has received little attention. Of the studies that have been carried out, Paul (1957), using a sclerotial homogenate, and Vining & Taber (1963), using a low-yielding surface culture of *Claviceps purpurea*, both demonstrated that alanine and phenylalanine were incorporated into the alkaloid, but the specificity of incorporation was not established. Majer *et al.* (1967) found that radioactivity from L-[U-¹⁴C]alanine was incorporated into the whole ergotamine molecule and only 12% of the label was associated with the α -hydroxy- α -alanine moiety. They could find no evidence for the incorporation of L-[U-¹⁴C]alaninol, which is in contrast to the findings of Minghetti & Arcamone (1969), who observed that L-alaninol was efficiently incorporated into the ergotamine. However, Minghetti & Arcamone (1969) did not investigate whether the carbon skeletons of alanine or alaninol were incorporated intact or whether only part of these precursors was utilized.

(I) Ergotamine

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Gröger & Erge (1970) reported that L-proline functioned as a precursor of the prolyl moiety of the side chain of the ergotamine alkaloids.

The present work is part of a study of the biosynthetic origin of the non-lysergic acid moiety of the peptide alkaloids, in this case ergotamine (Bassett *et al.*, 1971), with particular emphasis on the α -hydroxy- α -amino acid residue.

Materials and Methods

Chemicals

Chemicals used for the preparation of culture medium were obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K. Sucrose and citric acid were standard laboratory reagent grade and KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and aq. NH_3 solutions were A.R. grade. For scintillation counting, naphthalene (Micro-Analytical Reagent grade) was obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex, U.K.) and 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole was from CIBA (A.R.L.) Ltd., Rare Metals Division, Duxford, Cambridge, U.K. All other chemicals were obtained from British Drug Houses Ltd., Laboratory Chemicals Division, Poole, Dorset, U.K.

The silica gel used for t.l.c. was MN-silica gel-N, obtained from Macherey, Nagel and Co., Düren, Germany.

Alkaloid samples were kindly provided by Sandoz A.G., Basle, Switzerland. All radioactive compounds were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Fermentation procedures

Storage of C. purpurea for biochemical studies. The cultures of *C. purpurea* (Imperial College code IC/114/IRN and IC/117/2) were obtained by u.v. mutation and strain selection from the original isolate (IC/29/30) of Tonolo (1966). The organisms were grown in the seed-stage medium for 7 days, and then 10 ml of glycerol was added aseptically to the contents of the flask. A sample (approx. 1.5 ml) of the seed-stage suspension was then transferred, under sterile conditions, to an ampoule, which was flame-sealed. The ampoule was cooled to -70°C at a rate of about $1^\circ\text{C}/\text{min}$ and then transferred to a liquid N_2 tank for long-term storage.

For the preparation of cultures, the contents of the ampoule were slowly thawed and transferred totally to a seed-stage culture medium.

Culture media. Both the seed-state and the production-stage media used for these studies were modifications of the formulations developed by A. Tonolo (unpublished work). The seed-stage medium contained, per litre of tap water: sucrose, 100g;

$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.0g; KH_2PO_4 , 0.25g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.033g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.027g; yeast extract, 0.10g; L-cysteine hydrochloride, 0.10g; L-asparagine, 10g. The production-stage medium contained, per litre of tap water: sucrose, 300g; citric acid, 15g; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.0g; KH_2PO_4 , 0.5g; KCl, 0.12g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.007g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.006g.

The media were adjusted to pH 5–5.4 with 2.5M-NaOH for the seed-stage medium and with conc. aq. NH_3 (sp.gr. 0.88) for the production-stage medium. The culture fluid was autoclaved for 30 min at 35 kPa (5 lb/in²), giving a post-sterilization pH of 5.0–5.4.

All incubations were conducted in 500 ml cotton-wool-plugged Erlenmeyer flasks containing 100 ml of medium. The flasks were agitated on a rotary shaker (200 rev./min; 10 cm eccentric throw) in semi-darkness at 24°C . The seed-stage flasks were inoculated with the contents of one ampoule of stored mycelium and the production stages were inoculated with a 2 ml sample transferred directly from a seed-stage flask, after 7 days' growth.

Addition of labelled precursors. All solutions of labelled precursors were sterilized by filtration through a Millipore filter and added to the cultures under sterile conditions. The ^{14}C -labelled precursors were normally added to a single flask 9–10 days after inoculation and the culture broth was harvested after a further incubation of 6.5, 24 or 48 h.

For the studies of alanine incorporation, the precursor was added to duplicate cultures.

In the time-course experiments, 10–15 ml samples were withdrawn from the flasks at set intervals and analysed for total alkaloid content, total radioactivity content and pH of the medium. The alkaloid from these experiments was isolated (as shown below) and the total radioactive content measured.

Analytical procedures

Measurement of total alkaloid. The alkaloid concentration of aqueous solutions was assayed by the method of Voigt (1959).

Resolution of alkaloids. A t.l.c. system suitable for the resolution of the lysergic acid alkaloids was developed by a modification of the system of McLaughlin *et al.* (1964). T.l.c. plates were prepared on a spreader (TLC Apparatus, Quickfit Instruments Ltd.) with a 0.25 mm-thick layer for analytical work and a 0.75 mm-thick layer for preparative studies. The plates were air-dried for 15 min and then activated by heating at 105°C for 1 h. The solvent system used to develop the plates was ethyl acetate–*NN*-dimethylformamide–methanol (130:9:1, by vol.). The alkaloids were detected by their characteristic u.v. fluorescence and by the blue colour formed on spraying the chromatogram with 10% (w/v) *p*-dimethylaminobenzaldehyde in conc. HCl. Chromatograms were

developed in dark cupboards. R_f values were: lysergamide, 0.10; ergometrine, 0.17; *N*-(α -hydroxyethyl)-lysergamide, 0.22; ergotamine, 0.48; ergotoxine mixture 0.6–0.7. The C-8 epimers of the peptide alkaloids ran near the solvent front.

Determination of amino acids. Amino acids were separated and assayed by the fully automated procedure of Thomas (1970).

Measurement of radioactivity. Radioactive isotopes were assayed in a Packard Tri-Carb 3003 liquid-scintillation spectrometer. The scintillant system consisted of 30 g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole scintillant and 200 g of naphthalene dissolved to 5 litres in A.R.-grade toluene. The radioactivity of aqueous samples (0.5–1.0 ml) was determined in a mixture of 5 vol. of scintillant to 4 vol. of methoxyethanol (A.R.). A channels-ratio method was used to determine absolute radioactivity by using a quench-correction curve with standard [^{14}C]hexadecane and water as the quenching agent.

Isolation and purification of ergotamine

T.l.c. of the culture filtrate revealed that ergotamine was the major peptide alkaloid component, and smaller amounts of lysergic acid and members of the ergotoxine group, and their C-8 epimers, were also present. After being chilled, the culture filtrate was adjusted to pH 7.2–7.5 with aq. 2M- NH_3 and extracted three times with 1 vol. of ice-cold chloroform. The solvent was washed with aq. 1% (w/v) NaOH (3 $\times$ 1.5 vol.) and deionized water (4 $\times$ 1 vol.) to remove small amounts of ergochrome pigment, which were extracted into the organic phase. The chloroform extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* below 25°C in a rotary evaporator (Buchi, Rotavapor-R). The dried weight of the crude alkaloid was determined after drying in a vacuum desiccator over fresh P_2O_5 for 24 h, the normal yield being 12–18 mg/flask. Because of the instability of the alkaloid to light and heat, these and the following operations were carried out in a dimly lit cold-room or in flasks protected from light with aluminium foil.

Ergotamine was separated from the other alkaloids by the t.l.c. chromatographic system described above, in 'Analytical procedures', by using a 0.75 mm-thick layer of silica gel. Immediately after development, the stationary phase was dried in a stream of N_2 , in darkness, to remove the solvent. The area of the chromatogram corresponding to ergotamine was transferred to a vial and eluted with 3 $\times$ 10 ml of methanol–aq. NH_3 (sp.gr. 0.88) (20:1, v/v). The stationary phase was sedimented (3000 rev./min for 20 min; rotor no. 253, International PR-2 centrifuge) at 4°C and the combined supernatant was concentrated under vacuum at 30°C in a rotary evaporator. The alkaloid residue was dissolved in

10 ml of chloroform. For specific-radioactivity determinations of the undiluted alkaloid, 0.01–0.1 ml fractions were assayed for radioactivity, and 0.1 ml fractions were transferred to a volumetric flask; the solvent was removed in a stream of N_2 and the residue made up to volume (5 or 10 ml) in aq. 4% (w/v) tartaric acid for colorimetric determination (Voigt, 1959).

Degradation procedures

Ergotamine is readily degraded by both acid and alkali hydrolysis. Acid hydrolysis gives good yields of proline and phenylalanine from the side chain, but results in the destruction of the ergoline nucleus. The latter is recoverable as lysergic acid, after alkali hydrolysis (Jacobs & Craig, 1934, 1936–37; Smith & Timmis, 1937). After carefully controlled alkali hydrolysis, the α -hydroxy- α -amino acid moiety of the side chain of ergotamine may be isolated as the hydrazone of the corresponding α -oxo acid, albeit in fairly low yield (Stoll & Hofmann, 1950). Making the conditions for the recovery of this component optimum resulted in a diminution in the recoveries of phenylalanine and proline. After isolation of the alkaloid from the filtrate, the purified ergotamine was therefore divided into two separate portions for the performance of separate acid and alkali degradation. One portion was subjected to acid hydrolysis without addition of unlabelled carrier alkaloid, except in one experiment (Table 5). To the second portion unlabelled carrier ergotamine was added and subjected to alkali degradation to recover lysergic acid and pyruvate.

Acid hydrolysis. Ergotamine (0.5–2.5 μmol) was heated in 2 ml of 6M-HCl in an evacuated sealed tube at 120°C for 24 h and the hydrolysate was evaporated to dryness. Excess of HCl was removed by repeated addition of deionized water and re-evaporation. [^{14}C]Norleucine was added as a marker and the amino acids were separated by the fully automated rapid ion-exchange method of Thomas (1970). Analysis of the hydrolysate demonstrated, in addition to the added norleucine, the presence of phenylalanine, proline and an unexpected peak with a similar retention time to alanine.

It was usual to introduce between 0.5 and 2.5 μmol of amino acid on the column. The eluate from the column was divided into two accurately metered streams, one going to the analytical system and the other into the tubes of a fraction collector. The analytical system normally measured between 10 and 1000 nmol of each amino acid with an accuracy of $\pm 3\%$. Radioactivity was determined on the fractions containing the amino acids norleucine, phenylalanine, proline and alanine.

Alkali hydrolysis. In a typical experiment 160.3 mg (0.28 mmol) of ergotamine was recrystallized from

benzene (A.R.) yielding 149.7 mg (0.26 mmol), which was dissolved with gentle warming (25°C) in 2 ml of methanolic 1% (w/v) KOH (0.36 mmol). The methanol was removed on a rotary evaporator, leaving a glassy residue, which was dissolved in 2 ml of aq. 4% (w/v) KOH and heated under reflux in a stream of O₂-free N₂ in darkness for 40 min. The reaction mixture was rapidly cooled, immediately diluted with 2 ml of deionized water and adjusted to pH 8.0 with H₂SO₄.

Lysergamide and unchanged ergotamine are removed by extracting with 3 × 2 vol. of chloroform. The aqueous phase was neutralized with great care to pH 5.0 with 1 M-H₂SO₄, when colourless crystals of lysergic acid sulphate appeared, and these were collected after sedimentation at 4°C, washed with 0.5 ml of water and dried in a vacuum desiccator over fresh P₂O₅ overnight.

The lysergic acid sulphate was dissolved in 1.0 ml of methanol-aq. NH₃ (sp.gr. 0.88) (20:1, v/v) and applied along the centre line of a Whatman 3MM (57 cm × 45 cm) chromatography paper. The stationary phase was damped with buffer, pH 1.85, containing 78 g of acetic acid (A.R.) and 25 g of formic acid (A.R.)/litre. A potential of 3.5 kV was applied across the paper in a 10 kV Electrophoresis Unit (Miles Hivolt, Shoreham, Sussex, U.K.) giving a current of

400–480 mA. The potential was applied for 25 min and the free acid migrated towards the cathode. Lysergic acid was eluted from the paper in an analogous manner to the removal of ergotamine from silica gel; the amount of the ergoline derivative recovered was 26.48 mg (0.1 mmol, yield 38.3%).

Immediately after precipitation of lysergic acid sulphate, the reaction mixture, now at pH 5.0, was applied to a small (10 cm × 1.5 cm) Zeo-Karb 225 (H⁺ form) ion-exchange column. Pyruvate was eluted with 10 ml of deionized water and the eluate was collected in 10 ml of a solution containing 30 mg of *p*-bromophenylhydrazine hydrochloride and 20.5 mg of sodium acetate. The solution was warmed to 25°C for 10 min, acidified to pH 2.0 with 2 M-HCl and the precipitated hydrazone partitioned into ethyl acetate (4 × 10 ml). Shaking the organic solution with 4 × 10 ml of 10% (w/v) Na₂CO₃ returned the acidic hydrazone to the aqueous phase. The aqueous solution was cooled in crushed ice, carefully adjusted to pH 2.0 with HCl and the hydrazone extracted with 4 × 10 ml of ethyl acetate. The solution was dried over anhydrous Na₂SO₄ and then evaporated to dryness at 25°C in a rotary evaporator. The residue was dissolved in 1 ml of ethyl acetate and applied to the origin of a 0.75 mm-thick silica gel t.l.c. plate, which was developed in chloroform-methanol-acetic acid

Table 1. Incorporation of primary precursors of the ergoline system into the chloroform-extractable alkaloid fraction (mainly ergotamine)

All incubation experiments were carried out with *C. purpurea* strain IC/117/2P, except for the incorporation of sodium [2-¹⁴C]acetate, which was done with strain IC/114/1(RN). The precursors were added to single shake-flask cultures (100 ml), subject to a minimum alkaloid content of 250–350 µg/ml. The incorporation of radioactivity into peptide alkaloid is expressed as a percentage of the added precursor.

Expt. no.	Precursor (specific radioactivity)	Time of precursor addition (days)	Incubation time (h) after addition of precursor	Amount of precursor added (µCi)	Incorporation (%)
1	L-[methylene- ¹⁴ C]Tryptophan (54.5 mCi/mmol)	10	24	30	3.57
1a	L-[methylene- ¹⁴ C]Tryptophan (54.5 mCi/mmol)	10	48	20	10.93
2	DL-[2- ¹⁴ C]Mevalonic acid lactone (5.85 mCi/mmol)	10	24	25	5.3
2a	DL-[2- ¹⁴ C]Mevalonic acid lactone (5.85 mCi/mmol)	10	48	25	6.24
3	L-[methyl- ¹⁴ C]Methionine (10.2 mCi/mmol)	10	24	76	3.26
3a	L-[methyl- ¹⁴ C]Methionine (10.2 mCi/mmol)	8	48	20	1.21
4	Sodium [¹⁴ C]formate (10–50 mCi/mmol)	10	24	50	2.74
5	Sodium [2- ¹⁴ C]acetate (38.0 mCi/mmol)	10	24	15	2.43

(120:20:1, by vol.). The chromatogram was dried in a stream of N₂ and the hydrazone located by its u.v. absorption (R_F 0.82) and immediately eluted from the silica gel with 3 × 10 ml of aq. 95% (v/v) ethanol. The supernatant, after sedimentation of the stationary phase, was concentrated *in vacuo* at 30°C in a rotary evaporator and the residue made up to a known volume (2–5 ml). For specific radioactivity determination 0.1–0.5 ml fractions were assayed radiochemically, and the remainder was diluted for measurement of E at 325 nm. The concentration of pyruvyl-*p*-bromophenylhydrazone was determined from a standard graph.

Results

Table 1 lists the times of precursor addition, length of incubation and the percentage incorporation into ergotamine of the expected ergoline precursors, tryptophan, mevalonic acid, methionine and formate. Carbon atoms from all the precursors were readily incorporated into ergotamine. The incorporation from tryptophan after 48 h incubation was the highest, in accordance with studies of biosynthesis of other ergot alkaloids (Arcamone *et al.*, 1962; Agurell, 1966; Castagnoli *et al.*, 1970).

Degradation of the ergotamine isolated from the 24 h-incubation experiments (Table 2) confirmed that tryptophan, mevalonic acid and the methyl group of methionine specifically labelled the ergoline nucleus. Although lysergic acid was not isolated after incubation with [¹⁴C]formate, the facts that radioactivity was incorporated into the undegraded alkaloid to a considerable extent and that the side-chain amino acids, obtained after acid hydrolysis, contained only a small amount of radioactivity indicated that the ergoline nucleus could be the only site of labelling.

The structure of ergotamine suggested that the most probable precursors of the side chain of ergotamine are alanine, proline and phenylalanine. It was expected that the rate of metabolism of alanine would exceed that of proline and phenylalanine, thus resulting in the rapid elimination of labelled alanine by metabolic routes other than those involved in alkaloid biosynthesis. It was necessary therefore to establish the optimum times for the addition of each individual precursor. Time-course experiments, carried out with uniformly ¹⁴C-labelled amino acids, demonstrated that alanine was utilized from the culture broth at the fastest rate and was incorporated into the alkaloid at the most rapid rate. During 24 h incubation with alanine there was little increase in the radioactivity of the alkaloid after about 8 h, when approximately 0.10% of the feed precursor radioactivity was detected in the alkaloid fraction. The radioactivity of the ergot alkaloids produced in the presence of labelled proline and phenylalanine continued to increase for up to 48 h, when the values for

Table 2. Incorporation of specifically labelled precursors of the ergoline system into ergotamine

Expt. no.	Precursor	Specific radioactivity of ergotamine (μ Ci/mmol)	Radioactivity in each moiety (% of total parent alkaloid radioactivity)			
			D-Lysergic acid	Phenylalanine	Proline	Alanine
1	L-[methylene- ¹⁴ C]Tryptophan	54.89 2.35	— 95.3	0.92 —	0.093 —	0.12 —
2	DL-[2- ¹⁴ C]Mevalonic acid lactone	88.22 2.47	— 101.4	0.29 —	0.07 —	0.04 —
3	L-[methyl- ¹⁴ C]Methionine	36.64 0.87	— 93.1	0.59 —	0.39 —	1.12 —
4	Sodium [¹⁴ C]formate	21.26	n.d.	1.01	0.58	1.29

The upper-line value for specific radioactivity in each experiment refers to ergotamine isolated from the culture broth after chromatographic separation of the chloroform extract. The constituent side-chain amino acids phenylalanine, proline and alanine were assayed radiochemically after acid hydrolysis of a portion of the alkaloid. The lower-line value for specific radioactivity in each experiment refers to the remaining alkaloid after dilution with unlabelled carrier ergotamine (50–100 mg); this was then subjected to alkaline hydrolysis before the recovery of lysergic acid by paper electrophoresis. n.d., Not determined.

incorporation into the peptide alkaloid fraction reached 1% and 4% respectively. As a result of these time-course studies, incubations with proline and phenylalanine were continued for either 24 or 48 h, whereas incubations with alanine were terminated after either 6.5 or 24 h.

Conditions under which the labelled precursors were incubated with the organism and the percentage of the added label incorporated into ergotamine are shown in Table 3. The order of decreasing efficiency of incorporation of radioactivity into ergotamine was L-[U-¹⁴C]phenylalanine > L-[U-¹⁴C]proline > L-[U-¹⁴C]alanine > DL-[1-¹⁴C]alanine. The low incorporation of alanine is due to the multiplicity of metabolic demands on it.

The patterns of labelling after addition of radioactive proline and phenylalanine are shown in Table 4. Phenylalanine specifically labelled the phenylalanyl moiety of the side chain, negligible radioactivity being detectable only in either the other side-chain components or in the lysergic acid fragment. Proline was efficiently incorporated into the prolyl moiety, although some radioactivity appeared in the ergoline nucleus. There was a greater spread of radioactivity in the peptide alkaloid when L-[U-¹⁴C]alanine was used as the precursor (Table 5), which undoubtedly reflects the ease with which C-2 and C-3 of alanine are converted into other metabolites. The appearance of about 50% of the total radioactivity in the α -hydroxy- α -amino acid moiety strongly suggests that the carbon skeleton of alanine is the direct precursor. This was confirmed by the use of DL-[1-¹⁴C]alanine, when approx. 90% of the total radioactivity of ergotamine was recovered in the pyruvyl-*p*-bromophenylhydrazine derivative. The figures in the alanine column of Table 5 show the percentage of the total alkaloid radioactivity that was recovered in the alanine peak, after ion-exchange chromatography of an acid hydrolysate of radioactive ergotamine. Initially there was doubt about the origin of this alanine (see the Materials and Methods section). However, the good agreement between the percentage incorporation values into alanine obtained by the ion-exchange chromatography method and by direct precipitation of the *p*-bromophenylhydrazine (Table 5) strongly suggests that both originated from the α -hydroxy- α -alanine moiety of ergotamine.

Discussion

Tryptophan, mevalonic acid, formate and the methyl group of L-methionine were found to be specifically incorporated into the ergoline nucleus of ergotamine. These results were expected in the light of other work (Gröger *et al.*, 1960; Taylor & Ramstad, 1960; Birch *et al.*, 1960; Vining & Taber, 1963; Agurell, 1966; Majer *et al.*, 1967; Castagnoli *et al.*, 1970) and confirmed that the biosynthesis of

Table 3. Incorporation of L-[U-¹⁴C]phenylalanine, L-[U-¹⁴C]proline, L-[U-¹⁴C]alanine and DL-[1-¹⁴C]alanine into the peptide ergot-alkaloids produced by *C. purpurea* grown in submerged culture

Expt. no	Precursor (specific radioactivity)	Time of precursor addition (days)	Incubation time (h) after addition of precursor	Total precursor activity added (μ Ci)	Incorporation of radioactivity into alkaloid (% of precursor)
1a	L-[U- ¹⁴ C]Phenylalanine (10 mCi/mmol)	8	48	55	2.5
1b		10	48	25	5.03
1c		10	24	25	2.86
2a	L-[U- ¹⁴ C]Proline (7.8 mCi/mmol)	12	48	30	1.15
2b		10	48	25	2.74
2c		10	24	25	2.27
3a	L-[U- ¹⁴ C]Alanine (10 mCi/mmol)	11	24	30	0.26
3b		10	6.5	40	0.34
4a	DL-[1- ¹⁴ C]Alanine (22.1 mCi/mmol)	10	24	40	0.17
4b		10	6.5	40	0.065

Incubations with phenylalanine and proline were carried out with *C. purpurea* strain IC/117/2P, whereas all the alanine incubations were undertaken with strain IC/114/1(RN). The precursors were added at the later stages of the fermentation, subject to a minimal alkaloid content of 350–500 μ g/ml. Otherwise all experimental procedures were as described for Table 2.

Table 4. Biosynthesis of ergotamine, showing the distribution of radioactivity in the alkaloid after incubation with L-[U-¹⁴C]phenylalanine and L-[U-¹⁴C]proline

After incubation in the presence of the precursor the ergotamine was purified by t.l.c. and its specific radioactivity determined. The alkaloid was then chemically degraded under the following conditions. A, The ergotamine was diluted with 293 mg of unlabelled carrier alkaloid before both the acid and alkali hydrolyses. B, The undiluted ergotamine (1–3 μ mol) was hydrolysed in 2 ml of 6M-HCl at 120°C in a sealed tube for 18–24 h. The specific radioactivities of proline and phenylalanine were determined after ion-exchange analysis. C, The alkaloid not used for the acid hydrolysis was diluted with 140–160 mg of unlabelled carrier ergotamine, and subjected to alkali degradation. Pyruvate was isolated by direct precipitation as the *p*-bromophenyldiazotone derivative, and the lysergic acid was purified by paper electrophoresis.

Expt. no.	Conditions	Precursor	Specific radioactivity of ergotamine (μ Ci/mmol)	Distribution of radioactivity in the components of ergotamine (% of radioactivity of the parent alkaloid)				
				Lysergic acid	Phenylalanine	Proline	Pyruvate	
1a	A	L-[U- ¹⁴ C]Phenylalanine	1.06	2.78	96.1	4.6	0.2	
1b	B	L-[U- ¹⁴ C]Phenylalanine	61.36	—	93.64	1.03	—	
1c	B	L-[U- ¹⁴ C]Phenylalanine	44.12	—	95.60	1.42	—	
	C		0.29	2.08			0.2	
2a	B	L-[U- ¹⁴ C]Proline	11.51	—	8.1	77.8	—	
	C		0.12	14.4	—	—	2.22	
2b	B	L-[U- ¹⁴ C]Proline	33.26	—	2.8	78.5	—	
2c	B	L-[U- ¹⁴ C]Proline	27.23	—	1.34	74.4	—	
	C		0.11	21.5	—	—	1.82	

Table 5. Biosynthesis of ergotamine showing the distribution of radioactivity in the alkaloid after feeding with L-[U-¹⁴C]alanine and DL-[1-¹⁴C]alanine

The radioactive ergotamine was purified by t.l.c. and its specific radioactivity directly determined. The active alkaloid was degraded under the following conditions. A, The undiluted alkaloid (1–2 μ mol) was hydrolysed in 2 ml of 6M-HCl at 120°C. in an evacuated sealed tube, for about 24 h. The hydrolysate was made to a standard volume (1–4 ml) in 0.1 mM-norleucine containing 1.0 nCi of [1-¹⁴C]norleucine/ml. The constituent side-chain amino acids phenylalanine, proline and alanine were resolved by ion-exchange chromatography and their specific radioactivities determined. Although alanine was recovered in low yield (5–10%) the ¹⁴C radioactivity of the amino acid was measured to provide a comparison with the pyruvate obtained by direct precipitation as a substituted hydrazone. The alkaloid recovered after incubation with uniformly labelled precursor (Expt. 3a) was subjected to a duplicate set of acid hydrolyses to show the reproducibility of the method. B, The alkaloid not used for the acid hydrolysis was diluted with 110–160 mg of unlabelled carrier ergotamine and subjected to alkali degradation. Pyruvate was isolated as the *p*-bromophenylhydrazone and the lysergic acid was directly precipitated as the sulphate and purified by paper electrophoresis.

Expt. no.	Conditions	Precursor	Specific radioactivity of ergotamine (μ Ci/mmol)	Distribution of radioactivity in the components of ergotamine (% of radioactivity of the parent alkaloid)					
				Lysergic acid	Proline	Phenylalanine	Alanine	Pyruvate	
3a	A	L-[U- ¹⁴ C]Alanine	4.33	—	25.13	6.61	55.48		
	A		4.33	—	26.78	6.28	49.58		
3a	B		0.070	20.3	—	—		52.5	
3b	A	L-[U- ¹⁴ C]Alanine	5.63	—	21.8	5.21	56.3		
3b	B		0.146	23.10	—	—		54.7	
4a	A	DL-[1- ¹⁴ C]Alanine	2.92	—	4.7	1.63	84		
4a	B		0.097	12.9	—	—		89.6	
4b	A	DL-[1- ¹⁴ C]Alanine	1.84	—	6.4	1.76	94		

the lysergic acid moiety of ergotamine is similar to that found for the other ergot alkaloids.

Both L-[U-¹⁴C]phenylalanine and L-[U-¹⁴C]proline were found to be specific precursors for the constituent side-chain phenylalanine and proline moieties of ergotamine, confirming and extending the findings of Vining & Taber (1963) and Gröger & Erge (1970). The fact that proline was not quite so specific a precursor as phenylalanine probably reflected the ease with which proline enters the tricarboxylic acid cycle through ready conversion into glutamate and α -oxoglutarate; thus some random distribution of label supplied as proline may be expected. Abe *et al.* (1972a) have demonstrated that the L-phenylalanine-L-proline lactam was present in cultures of *Claviceps purpurea*, which accumulate ergotamine, and radioactive label from the lactam has been incorporated into ergotamine (Abe *et al.*, 1972b).

Nearly all the previous studies that have investigated the role of alanine as a possible precursor of the hydroxyamino acid fragment of the ergot alkaloids have utilized L-[U-¹⁴C]alanine as a precursor (Majer *et al.*, 1967; Gröger *et al.*, 1968; Nelson & Agurell, 1969; Castagnoli *et al.*, 1970). In each case the evidence was inconclusive, since with one exception (Gröger *et al.*, 1968) the radioactivity resident in the hydroxyamino acid moiety was less than 50% of the total parent alkaloid radioactivity: 57–71% with *N*-(α -hydroxyethyl)lysergamide (Gröger *et al.*, 1968), 40–41% with the same alkaloid (Castagnoli *et al.*, 1970), 37–45% with ergometrine (Nelson & Agurell, 1969), 12% with ergotamine (Majer *et al.*, 1967), approx. 38% with ergotamine and 26% for *N*-(α -hydroxyethyl)lysergamide, estimated by the present authors on the basis of specific radioactivity and alkaloid concentration (Minghetti & Arcamone, 1969). These results suggest that either alanine is not the direct precursor, but its carbon skeleton undergoes metabolic changes before being incorporated, or alanine is the direct precursor but is metabolized so rapidly that immediate equilibrium with a general metabolic pool is reached. Moreover, all the above experiments used an incubation period, in the presence of the precursor, which was relatively long, although it is known that alanine is actively metabolized by *Claviceps* (Gröger *et al.*, 1968), especially when added during the early stages of the fermentation process (Minghetti & Arcamone, 1969). In the present work a short incubation period was used and the precursors were added at a later stage of the fermentation, so as to minimize random distribution of radioactivity. Under these favourable conditions about half of the total alkaloid radioactivity was detected in the α -hydroxy- α -amino acid moiety.

The rapid random spread of the radioactivity presumably occurs via conversion of alanine into C₂ units and their subsequent involvement in the biosynthesis of the precursors of the ergoline nucleus and

of the side-chain proline. During this process C-1 of alanine is lost as CO₂, and thus takes no further major part in the metabolism of the organism. Further, serine derived biosynthetically from the intact carbon skeleton of DL-[1-¹⁴C]alanine is known to function as a precursor of tryptophan in *Claviceps* (Malin & Westhead, 1959), but the radioactivity of the incorporated tryptophan would be expected to be low, since the carboxyl group of the aromatic amino acid, corresponding to the carboxyl group of alanine, is lost in the biosynthetic pathway leading to the formation of the ergoline system (Gröger *et al.*, 1959). It would therefore be expected that very little random distribution of radioactivity from DL-[1-¹⁴C]alanine would appear in ergotamine. In fact, label from this precursor was found in ergotamine, and nearly all of the total alkaloid radioactivity was detected in the α -hydroxy- α -amino acid fragment of the molecule. This demonstrates, in conjunction with the L-[U-¹⁴C]alanine incubation results, that the whole C₃ skeleton of alanine is incorporated intact into this moiety. Contrary to the findings of other groups (Majer *et al.*, 1967; Nelson & Agurell, 1969), Minghetti & Arcamone (1969) found that with their strains of *C. purpurea* and *Claviceps paspali*, L-[U-¹⁴C]alaninol could function as a precursor of the hydroxyamino acid moiety of the lysergic acid alkaloids, but it was not determined whether it was utilized as a C₂ or C₃ fragment. Although it is now clear that an intact C₃ skeleton of alanine is incorporated, the exact nature of the direct precursor still remains to be elucidated.

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