[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY AND AGRONOMY, UNIVERSITY OF ILLINOIS AND THE ELI LILLY RESEARCH LABORATORIES]

Biosynthesis of Ergot Alkaloids[1]

BY R. J. SUHADOLNIK,[1] L. M. HENDERSON,[1] J. B. HANSON AND Y. H. LOO

RECEIVED JANUARY 20, 1958

The incorporation of DL-tryptophan, labeled with C^{14} in position 7a or the α-carbon atom, into the ergot alkaloids formed by *Claviceps purpurea* cultured on the host rye plant, was so low as to indicate that tryptophan is not a precursor of the lysergic acid moiety. Likewise the isotopes from C^{14}-labeled phenylalanine and acetate, as well as tritium-labeled anthranilic acid and tryptophan, were not incorporated to a significant extent into the alkaloids. A technique is described for the administration of possible precursors into the infected rye plants without subjecting them to soil bacteria or to the roots of the plant.

Five schemes have proposed that tryptophan or 5-hydroxytryptophan is the precursor of the indole moiety of lysergic acid,[2,3] which is the common hydrolysis product of at least six ergot alkaloids. None of these schemes has been supported by experimentation.

At the time these investigations were begun no convincing evidence of the production of lysergic acid alkaloids by the mold grown in artificial culture had appeared.[4-7] For this reason the work reported here employed cultures grown parasitically on Rosen rye. Various isotopically labeled, suspected precursors were injected into the peduncle of the plants bearing alkaloid-producing sclerotia. The isotope concentration in the ergonovine isolated was determined to obtain evidence bearing on the biosynthesis of lysergic acid.

Experimental

Cultivation of the Mold.—High ergot producing strains of *C. purpurea* were obtained through the courtesy of Dr. B. Malin, Eli Lilly and Company, Indianapolis, Indiana, Dr. R. W. Lewis, Michigan State University, East Lansing, Michigan and Dr. G. Gjerstad, University of Texas, Austin, Texas. Stock cultures grown on potato–dextrose agar could be stored for three weeks at 2°. Surface cultures of *C. purpurea* were grown on the medium of Berman and Youngken.[8]

Inoculation of Flowering Rye.—Tetraploid Rosen rye seeds were germinated and vernalized for 4–6 weeks at 3°. The plants were then grown in the greenhouse at moderate temperature and high humidity. In one experiment a plot was seeded in the fall and the resulting plants were used for experiments the following May. When the plants began to flower, they were infected by dipping or spraying the spikes with (1) an isotonic sucrose suspension of *C. purpurea* (Exp. 2 Eli Lilly Culture) or (2) spores taken directly from an agar slant and suspended in an isotonic sucrose solution (Exp. 3 Lewis Culture) or (3) spores taken directly from the infected plants (Exp. 4 Gjerstad Culture). The inoculum was applied to the plants every second day until the end of flowering (about 10 days).

Administration of Labeled Substrates.—The labeled substrates used were dissolved in isotonic saline or sucrose. When sclerotia appeared, usually 5–8 days after the first infection, 0.1 to 0.2 ml. of a solution of the labeled compound was injected with a No. 26 hypodermic needle into the upper internode of the plant. As the needle was removed, stopcock grease was applied at the site of the injection to prevent leakage. A small perforation was made in the stem a few inches below the spike to permit the escape of air displaced during the injection. The compounds were administered all in one dose or in 5 doses over a period of 5–10 days (Table I).

TABLE I

DISTRIBUTION OF THE C^{14} OF TRYPTOPHAN-7a-C^{14} IN RYE PLANTS INFECTED WITH *C. purpurea*[a]

Experiment	2[b]	3[c]	4[d]
Injected			
mg.	16	14.4	16
mμc.	12100	10890	12100
Sclerotia			
mg.	1822	1474	1638
mμc.	2300	1771	1950
% of C^{14} injected	19	17.2	16.2
Grain			
mg.	443	507	222
mμc.		805	
% of C^{14} injected		7.9	
Straw			
mg.	2268	2080	
mμc.	900	3200	
% of C^{14} injected	7.5	29	

[a] Specific activity of the tryptophan, 153 μc./m*M*. [b] Experiment 2, ten plants grown in greenhouse and infected with *C. purpurea* from Eli Lilly culture. The tryptophan was injected at one time. [c] Experiment 3, nine plants grown in the greenhouse and infected with culture obtained from R. W. Lewis. The tryptophan was injected five times. [d] Experiment 4, ten plants grown on an experimental plot and infected with culture obtained from G. Gjerstad. The tryptophan was injected five times.

Isolation of Ergonovine.—The sclerotia were harvested when they were highly pigmented and dry. This required about 3 weeks from the time of the first infection. Approximately 10 sclerotia, with an average weight of 15 mg., were obtained from each infected plant. The sclerotia were pooled, ground and extracted with hot ammoniacal–methanol (1:9) to a negative van Urk's test.[9] The extracts were taken to dryness and the resulting residue was extracted three times with 1% tartaric acid. The tartaric acid extract was adjusted to *p*H 8.5 with solid sodium carbonate and the solution saturated with sodium chloride. The alkaline–salt solution was then extracted three times with ethylene dichloride and the ergonovine estimated colorimetrically in an aliquot. After removal of the solvent this residue was dissolved in ether, carrier ergonovine was added followed by solid maleic acid, and the ergonovine maleate was allowed to crystallize at 4°. The product was recrystallized from methanol–ether. A portion of the alkaloid was chromatographed on Whatman No. 1 filter paper in butanol–water–acetic acid (4–5–1). The blue fluorescent spot of ergonovine was cut out, eluted with methyl alcohol and counted by

(1) This investigation was supported in part by a research grant (A-801) from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service. Present address: Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma.

(2) J. E. Saxton, *Quart. Revs.* (*London*), **10**, 108 (1956).

(3) A. Feldstein, *Experientia*, **12**, 475 (1956).

(4) G. Gjerstad and E. Ramstad, *J. Am. Pharm. Assoc.*, **XLIV**, 736 (1955).

(5) H. D. Michener and N. Snell, *Am. J. Botany*, **37**, 52 (1950).

(6) M. Abe, *et al.*, *J. Agr. Chem. Soc. Japan*, **29**, 697 (1955).

(7) W. A. Taber and L. C. Vining, *Can. J. Microbiol.*, **3**, 55 (1957).

(8) M. L. Berman and H. W. Youngken, *J. Am. Pharm. Assoc.*, **XVIII**, 200 (1954).

(9) H. van Urk, *Pharm. Weekblad.*, **66**, 473 (1929).

liquid scintillation reference counting. The scintillator used was 2,5-diphenyloxazole.

Isolation of Tryptophan from the Protein of the Sclerotia.—Two hundred seventy milligrams of dried, ground sclerotia was extracted three times with hot 10% trichloroacetic acid solution followed by three extractions with hot ethanol. The residue was autoclaved in a stainless steel beaker at 15 pounds pressure with 3 ml. of 3 *N* NaOH for 12 hr. The hydrolysate was adjusted to *p*H 7 with 2 *N* HCl and a known volume was removed for microbioassay of the tryptophan. Ninety-eight milligrams of carrier DL-tryptophan was added to the neutral solution and after it dissolved, glacial acetic acid was added to a concentration of 5%. The solution was passed through a partially deactivated carbon column[10] and developed with 100 ml. of an aqueous solution of 2% pyridine–5% acetic acid. The tryptophan was eluted with 90 ml. of an aqueous solution of 10% phenol–20% acetic acid. This eluate was extracted with ether and the aqueous fraction was taken to dryness. The dried residue was dissolved in 1 ml. of hot glacial acetic acid, an equal volume of hot benzene was added and the tryptophan acetate was allowed to crystallize overnight at 2° (yield 42 mg.). Thirty-seven milligrams of the tryptophan acetate was hydrolyzed by boiling with 2 ml. of water. The solution was treated with charcoal and the tryptophan crystallized after adding 4 ml. of ethanol (yield 14 mg.).

Preparation of Tritium and C^{14}-Labeled Compounds.—The tritium labeled anthranilic acid and tryptophan used in these experiments was prepared and purified to a constant specific activity according to the procedure of Wilzbach.[11] The tryptophan-7a-C^{14} was synthesized from aniline-1-C^{14},[12] The phenylalanine-1-C^{14} was generously supplied by Dr. R. F. Nystrom, the acetate-2-C^{14} by Dr. I. C. Gunsalus; the tryptophan-α-C^{14} was purchased from Tracerlab, Inc.

Counting Procedures.—The Packard Tri-Carb Liquid Scintillation counter was employed to count the ergonovine isolated from the sclerotia. All other C^{14} samples were combusted by the procedure of Van Slyke, *et al.*[13] The CO_2 was collected in an ionization chamber and counted with a vibrating reed electrometer. The tritium was counted by the procedure of Wilzbach, Kaplan and Brown.[14]

Results and Discussion

The results of three experiments with tryptophan-7a-C^{14} are shown in Table I.

The data show that the sclerotia from the three experiments contained an average of 17.7% of the administered isotope eleven days after the last injection of the tryptophan-7a-C^{14}. This high incorporation of C^{14} into the sclerotia suggested that a large part of the tryptophan-7a-C^{14} was translocated without degradation. To verify this hypothesis, the tryptophan was isolated from the protein in the sclerotia of exp. 4 and the C^{14} content determined. The specific activity of the tryptophan was 7.52 μc./m*M*. This represents a 2-fold dilution of this amino acid.

Incorporation of the Radioactivity into the Ergonovine.—The principal alkaloid found in rye ergot is ergonovine. The results of the incorporation of radioactivity into this alkaloid from tryptophan-7a-C^{14} are shown in Table II.

TABLE II

INCORPORATION OF RADIOACTIVITY FROM TRYPTOPHAN-7a-C^{14} INTO ERGONOVINE

Experiment[a]	2	3	4
Ergonovine			
mg. isolated	1.5	0.96	0.15
c./m./mg.	2400	2800	4800
specific activity (μc./m*M*)	0.68	0.81	1.3
Dilution	225[b]	189[b]	118[b]
			57.9[c]
% incorporation	0.014	0.012	0.003

[a] See Table I for culture and injection conditions. [b] Obtained by dividing the specific activity of the tryptophan-7a-C^{14} (153 μc./m*M*) by the specific activity of the ergonovine. [c] Obtained by dividing the specific activity of the tryptophan isolated from the sclerotial protein (75.2 μc./m*M*) by the specific activity of the ergonovine.

The evidence obtained under the conditions of these experiments (Table II) indicates that tryptophan is not directly concerned in the biosynthesis of the lysergic acid portion of ergonovine. The slight incorporation of radioactivity into the alkaloid is of the order of magnitude which might be expected from non-specific labeling from the products of tryptophan metabolism. Anthranilic acid and tryptophan, randomly labeled with tritium, tryptophan-α-C^{14}, acetate-2-C^{14} and phenylalanine-1-C^{14} were also studied as possible precursors of lysergic acid. From the results shown in Table III it is evident that an insignificant amount of the tritium and C^{14} was present in the alkaloid extract.

TABLE III

INCORPORATION OF RADIOACTIVITY INTO THE ERGOT ALKALOID EXTRACT[a]

Substrate	Amount injected, mμc.	Total alkaloid isolated, μg.	Alkaloid extract mμc.	Alkaloid extract % incorp.
Anthranilic acid-H^3	26000	21.4	1.5	0.006
Tryptophan-H^3	37000	35	1.2	.004
Tryptophan-α-C^{14}	23600	19.3	3.0	.012
Acetate-2-C^{14}	60000	28.6	0.3	.005
Phenylalanine-1-C^{14}	10000	7.1	0.3	.003

[a] The alkaloid content was too low to separate and isolate the ergonovine. Therefore, the calculations were based on total alkaloid.

This low incorporation of radioactivity into the crude alkaloid extract can be presumed to result from the products of degradation of the substrates by the fungus and not as the result of direct incorporation.

Acknowledgments.—The authors are indebted to Dr. Rice, Radiochemistry Department, Eli Lilly Company, for the C^{14} and tritium analyses of ergonovine and to Dr. R. F. Nystrom for assistance with the tritium labeling.

URBANA, ILLINOIS

(10) C. E. Dalgliesh, *J. Clin. Path.*, **8**, 73 (1955).

(11) K. E. Wilzbach, THIS JOURNAL, **79**, 1013 (1957).

(12) L. M. Henderson, R. D. Rao, R. F. Nystrom, "Biochem. Prep.," Vol. VI, in press, John Wiley and Sons, Inc., New York, N. Y.

(13) D. D. Van Slyke, R. Steele and J. Plazin, *J. Biol. Chem.*, **192**, 769 (1951).

(14) K. E. Wilzbach, L. Kaplan and W. G. Brown, *Science*, **118**, 522 (1953).