Effects of 5-Fluorotryptophan on the Biosynthesis of Ergot Alkaloids by *Claviceps purpurea*

By W. A. SKINNER, J. J. MORRIS, and J. V. STEVENSON

The possibility of incorporation of 5-fluorotryptophan into ergot alkaloids by *Claviceps purpurea* PRL 1565 was explored utilizing randomly tritiated material. Very little radioactivity was found in the alkaloid fraction, most of it residing either in the mycelium or in the base-soluble fraction which would contain unchanged 5-fluorotryptophan. Thin-layer chromatography allowed the isolation of an alkaloid containing radioactivity, but fluoride analysis using neutron activation indicated the absence of fluorine in the molecule. It was concluded that 5-fluorotryptophan was not incorporated into the ergot alkaloids produced by *C. purpurea* PRL 1565.

THE BIOSYNTHESIS of ergot alkaloids has been the subject of many studies (1–5), primarily with regard to the elucidation of the pathway followed and identification of precursors. Tryptophan is known to be actively incorporated into the ergot or clavine alkaloids by certain strains of *Claviceps*. However, except for two studies, ring-substituted tryptophans have not been investigated as precursors of the ergot nucleus. Arcamone *et al.* (6) investigated the effect of 4-,5- or 6-methyltryptophans on the ergot alkaloid production by *Claviceps paspali* Stevens and Hall in submerged culture. They concluded on the basis of comparison of chromatography of the products isolated from the media containing the 5- or 6-methyltryptophans with that containing tryptophan that no incorporation of the methyltryptophans had occurred. Further evidence cited for this was comparison of the infrared spectra of hydrazides of the *dl*-isolysergic acid obtained from the culture containing the 5- or 6-methyltryptophans with that of an authentic sample of the unsubstituted derivative. However, one would expect only small differences between the chromatographic behavior and infrared spectra of ring methyl substituted isolysergic acid amides and hydrazides and the nonring substituted analogs. The added 4-methyltryptophan inhibited alkaloid production, while the 5- and 6-methyl substituted tryptophans allowed production of alkaloids greater than the control without the addition of the tryptophans. This increase in alkaloid production could be due either to incorporation or enzyme induction.

The other study of the effect of substituted tryptophans in *Claviceps* on alkaloid production was conducted by Floss and Mothes (7) using a saprophytic culture of *Claviceps*-Stammes SD 58. These workers investigated the effects of 4-, 5-, 6-, and 7-methyltryptophans and 5-fluorotryptophan on alkaloid yield. The methyl ring-substituted tryptophans were all found to increase alkaloid production somewhat with the 5- and 6-isomers giving the greatest increase. A marked reduction in alkaloid yield was obtained with the addition of 5-fluorotryptophan but no incorporation data were obtained. The incorporation into elymoclavine of tritium marked 5- and 7-methyltryptophans was studied. No appreciable incorporation into elymoclavine was obtained, and the authors concluded that the increases in alkaloid production obtained with these ring methyl derivatives were due to enzyme induction.

In these laboratories, the authors have also been interested in whether ring-substituted tryptophans could be incorporated into ergot alkaloids as a possible biosynthetic method for the preparation of new ring-substituted ergot derivatives. The results of studies using randomly tritium-labeled 5-fluorotryptophan in submerged cultures of *Claviceps purpurea* PRL 1565 are reported.

METHODS

Two strains of *C. purpurea*, initially obtained from Dr. W. A. Taber, were used for experiments. Strain 1565 was found to give the highest yields of alkaloids when grown on a modified Abe medium plus yeast extract and glucose and strain 1578 when grown on Abe's medium plus ammonium succinate and a 19:1 mixture of galactose and glucose. The extraction procedure used was that of Vining and Taber (8). By using ergometrine as a control, the efficiency was determined to be satisfactory. Alkaloid content was ascertained by the van Urk method using ergometrine as a standard. The maximum alkaloid production was attained in 20–50 days. Addition of L-tryptophan, either just before the time of peak alkaloid production or earlier in the growth period, resulted in increased alkaloid production.

Investigation of various silica gel GF thin-layer chromatographic systems using authentic ergot and clavine alkaloids indicated that the two solvent systems, benzene–ethanol (2:1) and ethyl acetate–dimethylformamide–95% ethanol (10:1:1), were sufficient to separate most of the alkaloids of interest. Alkaloids could be detected under ultraviolet light or by spraying with *p*-dimethylaminobenzaldehyde reagent. In both solvent systems 5-fluorotryptophan and 5-fluorotryptamine stayed at the origin. In butanol–acetic acid–water (10:1:1), 5-fluorotryptophan has an R_f of 0.4.

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TABLE I—ALKALOID YIELDS PRODUCED BY *C. purpurea* PRL 1565

Expt.	Sample	Yields of Alkaloid, mcg./ml. 10 Days	15 Days	18 Days	20 Days	28 Days	35 Days
33	Control	8.5	...	...	8.5	...	9.0
	5-FT (labeled)[a]	2.5	...	...	2.5	...	19
	5-FT (labeled)[b]	6.5	...	...	12	...	15
36	Control	...	10	...	...	10	10
	5-FT (labeled)[c]	...	3.5	...	...	8.5	5.0
	5-FT (labeled)[b]	...	3.5	...	...	4.5	8.5
39	Control	...	9.5	10	10	10	...
	5-FT (labeled)[d]	...	...	2.0	3.5	5.0	
	5-FT (labeled)[b]	...	18	...	15	17	...

[a] Added on day 24 (1.5 mg. with specific activity of 2.46×10^7 d.p.m./mg.). [b] In addition to labeled 5-FT added on day 24, L-tryptophan added at time of inoculation to yield final concentration of 0.1%. [c] Added on day 6 (1.1 mg. with specific activity of 2.46×10^7 d.p.m./mg.). [d] Added on day 4 (1.3 mg. with specific activity of 2.34×10^7 d.p.m./mg.).

RESULTS

5-Fluorotryptophan (K and K Chemical Co.) was identified by its melting point and infrared absorption spectrum and randomly tritiated at the New England Nuclear Co. by the Wilzbach technique of exposure of 375 mg. at 250 mm. pressure to 3 c. of tritium at 27° for 2 weeks. The labile tritium was removed by dissolving the material in 95% ethanol and by removal of the solvent *in vacuo*. This material was purified by crystallization from isopropanol–water to yield material whose infrared absorption spectrum and thin-layer behavior in butanol–acetic acid–water (10:1:1) and in *n*-propanol–concentrated ammonium hydroxide (70:30), R_f 0.5, agreed with that of authentic material. This purified material, which also showed no change in specific activity upon recrystallization, was added to submerged cultures of *C. purpurea* PRL 1565 and alkaloidal yields were determined colorimetrically by using van Urk reagent (Table I). In some experiments L-tryptophan was added at the time of inoculation and the labeled 5-fluorotryptophan was added later.

TABLE II—DISTRIBUTION OF RADIOACTIVITY IN EXTRACTS FROM *C. purpurea* PRL 1565

Expt.	% of Counts Basic Extract	Neutral Extract	Acidic Extract	Mycelium
33	20	8.2	5.0	66.8
36	33.6	0.8	3.0	62.6
39	10.6	7.5	7.7	74.2

TABLE III—THIN-LAYER CHROMATOGRAPHY OF ALKALOIDS FROM *C. purpurea* PRL 1565 AND STANDARD ALKALOIDS

Alkaloid Extract	Ethyl Acetate–Dimethylformamide–95% Ethanol (10:1:1) Orig., 0.55, 0.90	Benzene–Ethanol (2:1) Orig., 0.60, 0.90
Chanoclavine	0.05	0.06
Ergonovine	0.17	0.20
Agroclavine	0.40	0.44
Ergosine	0.54	0.48
Ergotamine	0.55	0.65
Ergocristine	0.58	0.70
Ergocryptine	0.60	0.75
Ergocornine	0.68	0.70
Ergocristinine	0.75	0.70
Ergotaminine	0.90	0.85

Distribution of the radioactivity among extracts from the various experiments is shown in Table II. Each per cent represents the average of results from four separate flasks.

Investigation of the thin-layer behavior (silica gel GF) of the acidic extracts after basification with ammonium hydroxide and extraction of the alkaloids with $CHCl_3$ and ether gave results as seen in Table III when compared to authentic samples of ergot and clavine alkaloids. These R_f values are averages of a large number of determinations.

The spots were all detected by their fluorescence and all but the origin spots were van Urk positive. The alkaloid at 0.55 in ethyl acetate–dimethylformamide–95% ethanol and 0.60 in benzene–ethanol corresponds most closely to ergotamine. The alkaloid at 0.90 corresponds most closely to ergotaminine.

The alkaloid mixture isolated from experiment 39 (Table II) was put on a thick-layer silica gel GF plate and developed with ethyl acetate–dimethylformamide–95% ethanol (10:1:1). The plate was sectioned to separate the fluorescent spots at the origin, 0.55 and 0.90 and the silica gel was extracted, first with methanol, then with chloroform. The combined extracts were dried *in vacuo* and counted in a scintillation counter. The products had the following counts: origin (8,040 c.p.m.), 0.55 (92,300 c.p.m.), and 0.90 (6,390 c.p.m.). Nonfluorescent sections between these zones had only background counts.

The van Urk positive radioactive material at 0.55 was isolated from a silica gel H plate by removal of the zone of ultraviolet absorption and extraction with methanol. The methanol was removed *in vacuo* and the sample submitted along with a control sample to General Atomic Division of General Dynamics, San Diego, Calif., for fluoride analysis by neutron activation. The results were 1.1 ± 0.2 mcg. F for the blank and 0.7 ± 0.3 mcg. F for the alkaloid extract.

DISCUSSION

5-Fluorotryptophan was found to somewhat inhibit production of ergot alkaloids from *C. purpurea* PR 1565 when added early after inoculation, agreeing with the results that Floss and Mothes found on clavine alkaloid yields using *Claviceps*-Stammes SD 58. However, when the 5-fluorotryptophan was added on the day 24 after inoculation, the alkaloid yields were increased.

The distribution of radioactivity after addition of

randomly tritiated 5-fluorotryptophan to *C. purpurea* PRL 1565 was found to be largely in the mycelium and the basic extract which would contain unincorporated labeled 5-fluorotryptophan. In the alkaloid containing extract, the majority of the radioactivity was found to be concentrated in the thin-layer chromatographic spot at R_f 0.55 when developed with ethyl acetate–dimethylformamide–95% ethanol (10:1:1). The low yields of alkaloids produced by the organism prevented conventional fluoride analyses on them. The sensitivity of neutron activation analysis for fluoride was useful in indicating the absence of fluorine in the radioactive, van Urk positive material. The conclusion is that 5-fluorotryptophan was not incorporated into the ergot alkaloids by *C. purpurea* PRL 1565. Two possible explanations could account for the radioactivity of the alkaloid at R_f 0.55: tritium exchange between the tritiated 5-fluorotryptophan and tryptophan or enzymatic reduction of the 5-fluoro group followed by incorporation of the tryptophan. Doubly labeled 5-fluorotryptophan with a better alkaloid producing organism would be required to distinguish between these explanations.

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Gastrointestinal Irritant Effect of Glycerin as Compared with Sorbitol and Propylene Glycol in Rats and Dogs

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Effects of glycerin, sorbitol, and propylene glycol on the gastrointestinal mucosa were compared in rats and dogs. At equivalent, undiluted doses (ml./Kg.) in both species glycerin was more irritating than sorbitol, and propylene glycol was the least irritating of the three compounds studied. The types of irritant effects observed were qualitatively similar, and the degree of severity was dose-dependent in both species. Studies in rats showed that irritation produced by any of the three compounds was reduced by dilution of the dose.

GLYCERIN is a fairly common component of liquid pharmaceutical vehicles. However, there is little definitive information in the literature on its gastrointestinal effects. Johnson *et al.* (1) reported that ingestion of 30 ml. (approximately 0.6 ml./Kg.) of 95% glycerin with orange juice three times daily for 50 days by 14 normal humans produced no consistent changes in body weight or temperature and no overt pharmacological effects. The daily number and consistency of stools was unaffected by the quantities of glycerin fed. Subjectively, four subjects (29%) reported an occasional "sensation of warmth" in the stomach after ingestion, especially in the rare instance when glycerin was taken on an empty stomach. No enterogastric examinations were done, however, to assess gastrointestinal effects directly. These same investigators noted "no ill effects" in 11 fasted rats which received single oral doses of glycerin ranging from 7 to 18 Gm./Kg. (5.6 to 14.3 ml./Kg.). Arnschnik (2) cited "no illness" in dogs after single oral doses of glycerin up to 8 Gm./Kg. (6.3 ml./Kg.). However, at 11 Gm./Kg. orally (8.7 ml./Kg.), the same investigator reported "intestinal disturbances" and vomiting. Kobert (3) and Sollmann (4) stated that repeated ingestion of large doses of glycerin lead to a chronic inflammation of the gastrointestinal tract in man. Deichmann's review of the pharmacology of glycerin (5, 6) cited Kobert's report (3) of the accidental ingestion of 300 Gm. (about 238 ml.) of glycerin by a 2-year-old child who lost consciousness but recovered following stomach lavage. Drill (7) noted that oral administration of 100 to 300 ml. of undiluted glycerin has caused "severe symptoms" in humans, whereas the same amount diluted or mixed with food produces "little or no effect." Sroka (8), in a patient given "more than 100 grams orally" while being treated for kidney stones, observed dizziness, drowsiness, violent headache, bloody diarrhea, and kidney pains (differing from the orginal kidney pains).

The purpose of this study was twofold: (*a*) to compare the gastrointestinal irritation liability of glycerin with that of sorbitol (in aqueous solution) and propylene glycol, two other commonly used constituents of oral liquid pharmaceutical preparations; and (*b*) to determine the effect of water dilution on the gastrointestinal irritant potential of these three compounds.

METHODS

Female Charles River rats, weighing 150 to 210 Gm., and adult mongrel dogs of either sex, weighing 8.9 to 16.0 Kg., were used. The rats were housed five per cage, and the dogs caged individually, in rooms maintained at 74° F. The compounds studied (glycerin, sorbitol, and propylene glycol) were administered by stomach tube, three times

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