Physiological Studies on Ergot: Influence of 5-Methyltryptophan on Alkaloid Biosynthesis and the Incorporation of Tryptophan Analogs into Protein

J. E. ROBBERS and H. G. FLOSS*

Abstract □ Time-course studies were performed which compared the influence of 5-methyltryptophan to tryptophan on alkaloid formation in ergot strain SD-58. Evidence was obtained that confirms the role of tryptophan as an inducer of alkaloid synthesis. Additional studies were conducted to determine the incorporation into protein of the tryptophan analogs: 5-methyltryptophan, 7-methyltryptophan, and 1-*N*-methyltryptophan, when they are supplied exogenously to the fungus. Incorporation was observed; however, it was insignificant when compared to the amount of tryptophan incorporated.

Keyphrases □ Ergot alkaloid production—shake cultures □ Tryptophan, 5-methyltryptophan effect, alkaloid production—comparison □ Protein incorporation—tryptophan and analogs

The important role of tryptophan in ergot alkaloid biosynthesis has been clearly established. Floss and Mothes (1) have obtained data which suggest that tryptophan is also involved in an induction phenomenon besides serving as a precursor in the formation of the ergoline ring system. These workers found that tryptophan stimulates alkaloid formation when added at the beginning of the culture period but not when added after alkaloid production had started, that a variety of tryptophan analogs stimulate alkaloid production even though they are not incorporated into the alkaloids, and that mycelia grown in the presence of tryptophan or its analogs retain the ability to produce more alkaloid than the controls even after replacement into fresh culture medium.

One of the disadvantages of this earlier work was that the percentage increase in alkaloid production due to tryptophan analogs was small because these experiments were carried out with cultures that produced large amounts of alkaloids. By using a fully synthetic culture medium, it was thought that the effect of tryptophan and its analogs could be demonstrated more dramatically. Eliminating yeast extract from the medium reduces the growth of the organism to about one-fourth that of the complete medium; however, the amount of alkaloid produced per gram of mycelium is unchanged.

The purposes of this investigation were to study the induction phenomenon in a more precise manner over the time period in which alkaloid production is initiated in the fungus and to compare the effect of 5-methyltryptophan with that of tryptophan.

In the series of experiments previously mentioned, Floss and Mothes (1) also found uptake into the mycelia of radioactivity from the methyltryptophan analogs. However, the metabolic fate of the analogs was not determined except for nonincorporation into alkaloids. Recent studies by Lark (2) have shown that *Escherichia coli* can incorporate 5-methyltryptophan into protein. Consequently, the present authors have attempted to determine if methyl analogs of tryptophan are incorporated into protein by the ergot fungus.

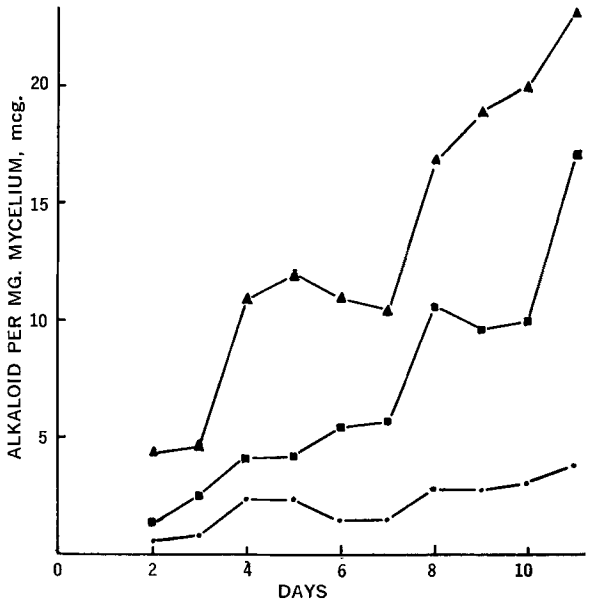

Figure 1—*Time-course study comparing the influence of tryptophan and 5-methyltryptophan on alkaloid production in* Claviceps *strain SD-58. Key:* ●, *control;* ▲, *tryptophan; and* ■, *5-methyltryptophan.*

EXPERIMENTAL

Induction of Alkaloid Production—*Claviceps* strain SD-58 was grown for 5 days in shake culture in a medium containing the following ingredients: 50 g. mannitol, 50 g. sucrose, 5.4 g. succinic acid, 3.0 g. Difco yeast extract, 0.1 g. KH_2PO_4, 0.3 g. $MgSO_4 \cdot 7H_2O$, 0.0044 g. $ZnSO_4 \cdot 7H_2O$, and boiled tap water, 1 l. The mycelia from one culture were placed into 0.01 *M* phosphate buffer and resuspended. Using aliquots of this suspension as inoculum, three groups of flasks containing the inoculum medium without yeast extract were prepared. One group of cultures also contained 4×10^{-3} *M* DL-tryptophan and a second group contained 4×10^{-3} *M* DL-5-methyltryptophan. A flask from each of the three groups was harvested every day starting at the 2nd day of growth. Quantitation of the alkaloids in the culture filtrate was effected by extracting the alkaline culture filtrate with $CHCl_3$ and redissolving the dried $CHCl_3$ extract in 2% succinic acid solution. This procedure prevented tryptophan and 5-methyltryptophan from interfering in the quantitation. A colorimetric determination was performed using van Urk's reagent (3, 4).

Analog Incorporation into Protein—Ergot strain SD-58 was grown in shake culture in the mannitol–sucrose–succinic acid–yeast extract medium previously described. Tritiated tryptophan, 5-methyltryptophan, 7-methyltryptophan, and 1-*N*-methyltryptophan were added, one to a culture, at the start of the growth period. The tritiated compounds were from the same source as in a previous study (1). On the 3rd and 6th days of growth the mycelia were removed by filtration and dried. The protein was extracted from the mycelia using trichloroacetic acid precipitation methods (5) and quantitated with the biuret reaction. A second purification was effected by redissolving the protein in 1 *N* NaOH and repeating the

Table I—Incorporation of ^{3}H-Labeled Tryptophan and Methyltryptophans into Elymoclavine and Protein

	Tryptophan	5-Methyl-tryptophan	7-Methyl-tryptophan	1-*N*-Methyl-tryptophan
Specific activity of added compound	7.0 mc./mmole	1.27 mc./mmole	0.39 mc./mmole	1.1 mc./mmole
Total activity fed	2.8 μc.	1.91 μc.	1.93 μc.	1.82 μc.
Absolute incorporation into alkaloids	35.3%	0.9%	0.2%	1.8%
Absolute incorporation into protein, 3rd day	39.6%	0.3%	0.7%	0.5%
Absolute incorporation into protein, 6th day	5.0%	0.8%	0.8%	0.5%

precipitation and quantitation methods. Using a measured amount of the repurified protein, which was dissolved in a 1 *N* NaOH solution, the incorporation of radioactivity into the protein was determined.

Approximately 50 mg. of protein powder obtained from each culture was hydrolyzed for 24 hr. at 125° with a saturated solution of barium hydroxide in an evacuated sealed glass tube. The barium was removed from the hydrolysate through the addition of 2 *N* sulfuric acid to pH 6 and subsequent centrifugation to remove the barium sulfate.

To investigate the presence of tritiated tryptophan and tryptophan analogs in the protein hydrolysates, ascending paper chromatography in *n*-butanol–acetic acid–water (4:1:1) was employed. By cutting the chromatogram into 1-cm. horizontal strips starting at the point of origin, placing these strips in toluene scintillation counting solution, and counting the radioactivity, the zones of activity on the chromatograms were determined, and the R_f values were compared with reference compounds.

Elymoclavine was isolated from 6-day-old culture filtrates by partitioning and recrystallizing from methanol to a constant specific radioactivity.

RESULTS AND DISCUSSION

The time–course study showing that tryptophan and 5-methyltryptophan stimulate alkaloid production early in the growth phase of the fungus (Fig. 1) confirms and extends earlier observations (1). Under the modified conditions, the effect of 5-methyltryptophan can be seen more clearly than in the earlier experiments. At Day 11, tryptophan induces a 495% increase and 5-methyltryptophan a 341% increase in alkaloid production over the control culture, whereas the previous work (1) shows only a 61% increase in alkaloids for each compound after 22 days. The time–course experiment was repeated four times and the results were found to be reproducible. Figure 1 represents the results from a single experiment. Stimulation of alkaloid production in *Claviceps* has also been reported by Baxter and Zahid (6). These data give supportive evidence to the postulation (1) that tryptophan is an inducer of alkaloid biosynthesis.

An explanation of this phenomenon becomes more difficult when one considers the work recently reported by Lingens *et al.* (7, 8) on the regulation of aromatic amino acid biosynthesis in *Claviceps*. Using alkaloid-producing mycelia of a strain of *Claviceps paspali*, they found (7) that L-tryptophan inhibited one of the isoenzymes of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) synthetase, reversed the inhibition of chorismate mutase by phenylalanine and tyrosine, and also activated this enzyme; but unlike the situation in other microorganisms, it did not inhibit anthranilate synthetase. 5-Methyltryptophan elicited the same effect as tryptophan on DAHP synthetase and apparently (8) also on chorismate mutase. In similar studies with *Claviceps* strain SD-58, however, it was found that anthranilate synthetase was inhibited by tryptophan (8). A weak point of the latter result is that it was not known whether the mycelium produced alkaloids under the culture conditions used.

The results clearly show that in strain SD-58 the addition of 5-methyltryptophan leads to increased alkaloid production. On the other hand, the incorporation study shows that 5-methyltryptophan cannot replace tryptophan as alkaloid precursor (Table I). Likewise, the organism can grow in the presence of 5-methyltryptophan without added tryptophan, yet 5-methyltryptophan is not incorporated into protein to any significant extent (Table I). This leaves unexplained the source of the endogenous tryptophan. One simple explanation would be that in ergot strain SD-58, 5-methyltryptophan cannot replace tryptophan as feedback regulator of aromatic amino acid biosynthesis. As another possibility, an anthranilate synthetase isoenzyme could exist in the fungus which is not inhibited by tryptophan or 5-methyltryptophan.

There is a low level of incorporation of radioactivity into protein when tritiated methyl analogs of tryptophan are fed to ergot strain SD-58 (Table I). However, their absolute incorporation appears insignificant when compared to the incorporation of tryptophan (approximately 80 times greater in the 3-day-old cultures). The decreased incorporation into protein of labeled tryptophan between Day 3 and Day 6 can be attributed to a dilution effect caused by the synthesis of nonlabeled endogenous tryptophan and has been observed before (5). Because of this low level of incorporation, it was not possible to determine conclusively if the activity was due to the direct incorporation of the analogs or if the analogs were metabolized to intermediates that could be utilized in amino acid biosynthesis and thus be incorporated into protein in this manner. However, the results from the chromatographic investigation of the protein hydrolysate tend to support the former viewpoint.

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