END-PRODUCT REGULATION OF ERGOT ALKALOID FORMATION IN INTACT CELLS AND PROTOPLASTS OF *CLAVICEPS* SPECIES, STRAIN SD 58.

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ABSTRACT.—Inhibition of alkaloid synthesis by elymoclavine was demonstrated in cultures of intact cells and in protoplast suspension of *Claviceps* strain SD 58. Assays of dimethylallyltryptophan (DMAT) synthetase in rigorously dialyzed cell-free extracts from protoplasts showed that the level of enzyme present was not significantly affected by exposure of the protoplasts to elymoclavine for 4 to 24 hours. These results suggest the operation of feedback inhibition, but probably not end-product repression, in ergot alkaloid biosynthesis in *Claviceps* strain SD 58.

The biosynthesis of the medicinally important ergot alkaloids has been studied extensively in intact cells of various *Claviceps* species and, to a lesser extent, with isolated enzymes from *Claviceps* mycelia (1). This has led to the establishment of the origin of the ergoline ring system from tryptophan and mevalonate and a methyl group from methionine, the elucidation of many aspects of the biosynthetic pathway, and the isolation of a few of the enzymes involved in this biosynthesis. In addition, a substantial amount of work has been done on the physiology and regulation of alkaloid formation in cultures of *Claviceps* (2, 3) by a number of laboratories. Work along these lines in our laboratory has focused largely on the identification of regulatory principles which operate in the control of alkaloid formation in ergot fermentations. We have demonstrated that alkaloid formation is subject to some form of induction by one of the substrates, tryptophan (4). Dimethylallyltryptophan (DMAT) synthetase which catalyzes the isoprenylation of tryptophan, the first committed reaction in the ergoline biosynthetic pathway, has been highly purified (5). During studies on this enzyme, it was observed (2, 6) that agroclavine and elymoclavine, the terminal alkaloids in the ergot strain used for the enzyme isolation, inhibited the purified enzyme 90% and 70%, respectively, at concentrations of 3 mM (750 mg/liter). In addition, *in vitro* studies have demonstrated inhibition by elymoclavine of chanoclavine-I cyclase which catalyzes the conversion of chanoclavine-I into agroclavine (7) and of anthranilate synthetase (8). From these results, the question can be posed as to whether or not the observations on isolated enzymes have any physiological significance and whether end-product control of alkaloid synthesis occurs *in vivo*.

In this paper we present data which indicate feedback inhibition under physiological conditions of dimethylallyltryptophan synthetase, the first enzyme in the biosynthetic sequence, by elymoclavine an end product of the pathway. In addition to using intact cells, we demonstrated alkaloid synthesis in protoplasts of *Claviceps* species, strain SD 58, and used this system to examine the significance of end-product regulation *in vivo*.

MATERIALS AND METHODS

ORGANISM AND CULTURING PROCEDURES.—The strain of ergot used for this study was *Claviceps* species, strain SD 58 (ATCC 26019) and was originally isolated from sclerotia obtained from the host *Pennisetum typhoideum* Richard.

The organism was maintained on Czapek-Dox agar slants and grown in shake cultures at 25° and at 180 rpm on a New Brunswick model G54 rotary shaker. Cultivation of the strain

was performed by inoculation of 500-ml Erlenmeyer flasks containing 100 ml of culture medium NL-406 (4) with a portion of the mycelium from an agar slant.

After ten days, 2 ml of the shake culture was transferred to fresh NL-406 medium, and this was repeated (usually four times) until the culture was homogenous in appearance.

For preparation of stationary cultures a 2-ml aliquot was transferred into 100 ml of NL-406 medium in 500-ml Fernbach flasks and allowed to grow at 24°.

To replace a growing culture, one culture was filtered through a sterile coarse sintered glass funnel. The mycelial pad was washed three times with small volumes of sterile doubly distilled water. With a sterile spatula, the mycelial pad was transferred to flasks containing appropriate replacement medium. To replace the media in the stationary cultures a sterile needle (3.5 in, 15 gauge) was inserted aseptically through the mycelial mat; the old medium was removed with a syringe and discarded. Another sterile needle was used to inject the fresh medium through the same hole in the mat.

Shake cultures were harvested by passing the suspension through a Whatman No. 1 filter paper on a Buchner funnel. The mycelial mats were washed with doubly distilled water and the total volume of the filtrate was determined. Stationary cultures were harvested by decanting the medium; the mycelia were removed with a spatula and washed with doubly distilled water.

PROTOPLAST FORMATION.—Using the method of Stahl *et al.* (9), mycelium from 3- to 5-day-old shake cultures was harvested on a coarse sintered glass funnel and washed three times with a small volume of 0.7 M KCl solution. After being suspended in 60 ml of 0.7 M KCl stabilization solution containing 6.7 ml liquid β-glucuronidase/arylsulfatase lytic enzyme (from *Helix pomatia*, Boehringer Manheim Biochemicals), the mycelium was incubated with shaking at 28° to 30° for 4 hr. The protoplast solution was centrifuged at 4,500 x *g* for 10 min. The protoplast residue was washed twice with 0.7 M KCl solution and centrifuged at 4,500 x *g* for 10 min. The residue was then placed into 0.7 M KCl solution and resuspended. Protoplasts were observed with a Zeiss model RA38 microscope at 1000 magnification.

REGENERATION OF INTACT CELLS FROM PROTOPLASTS.—To carry out the regeneration of intact cells, protoplasts were plated onto agar plates containing a medium composed of 5% beerwort (courtesy of Anheuser-Busch Inc., St. Louis, MO), 0.3% yeast extract (Difco), 2.5% agar at a pH of 5.5. After 3 to 5 days the regenerated cells were transferred to NL-406 medium and grown in shake cultures. Another method involved transferring protoplasts directly into NL-406 shake cultures.

In vivo INHIBITION EXPERIMENTS.—For the experimental flasks, 80 ml of NL-406 culture medium which contained the amount of ingredients required for 100 ml of medium was placed in a 500-ml flask and the pH was adjusted to 5.4 with concentrated NH_4OH. An elymoclavine succinate stock solution was prepared by dissolving elymoclavine and succinic acid (mole ratio at 2:1) in the desired amount of doubly distilled water. This solution was sterilized by passing through a membrane filter (Millipore Corp.) into a sterile flask. Varying concentrations of elymoclavine succinate were obtained in the experimental culture by using aliquots of the stock solution and appropriate amounts of ammonium succinate or potassium succinate in all of the cultures as well as a 100-ml final volume.

For control cultures, an appropriate amount of ammonium succinate or potassium succinate solution was added to NL-406 medium to provide an equivalent amount of succinate as in the experimental flasks.

For the inhibition study in protoplasts, protoplasts were suspended in 0.7 M KCl stabilizing solution. When elymoclavine succinate was added to the experimental flasks, the isotonicity of the stabilizing solution was maintained by the addition of variable amounts of KCl.

In both intact cell and protoplast experiments, inhibition of alkaloid biosynthesis by elymoclavine was determined by measurement of either differences in the incorporation rate of L-[methylene-^{14}C] tryptophan into the alkaloids or of differences in DMAT synthetase activity as compared to controls in which elymoclavine had not been added.

DETERMINATION OF % INCORPORATION OF TRYPTOPHAN INTO ALKALOID.—L-[methylene-^{14}C] tryptophan, 0.5 μCi for stationary cultures, 1 μCi for shake cultures, and 2 μCi for protoplast incubations, was added to cultures and incubation mixtures in an aqueous solution which was sterilized by passing through a membrane filter (Millipore Corp.). The alkaloid titer in aliquots of culture filtrates and protoplast incubations was determined before extraction. The aliquots were then made alkaline to pH 10 with NH_4OH and the total alkaloids were extracted three times with chloroform. The alkaloidal extract was further extracted three times with 2% succinic acid aqueous solution. The acid was made alkaline to pH 10 with NH_4OH, and the alkaloids were extracted three times into chloroform. The combined chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under vacuum. After the extracted alkaloids were dissolved in absolute methanol, a portion was used to determine alkaloid titer and a second portion was taken for radioactivity determination in a scintillation counter.

Alkaloids were quantitated in micrograms using the modified Van Urk method (10, 11).

In this procedure, after 20 min the optical density at 580 nm was determined for the assay mixture. This result was multiplied by 46.2, which is the reciprocal of the slope of a standard curve based on elymoclavine, the predominant alkaloid produced by *Claviceps* strain SD 58.

The percent incorporation is defined as the percent of radioactively labeled precursor that ends up in the total alkaloid.

ENZYME ASSAYS.—Powdered, lyophilized mycelium (250 mg) was mixed with 10 ml of 0.01 M Tris·HCl buffer (pH 8.0) containing 20 mM sodium diethyldithiocarbamate, 20 mM sodium thioglycolate, 20 mM 2-mercaptoethanol, 20 mM calcium chloride and 10% glycerol (5) and stirred for 2 hr at 0° to 4°. The reconstituted mycelium was centrifuged at 27,000 x *g* for 20 min to remove cellular debris and intact mycelium. The supernatant was termed the crude enzyme extract.

In the preparation of cell-free extract from protoplasts, the protoplast residue was treated in the same manner as the powdered mycelium with the exception that the mixture was centrifuged at 18,000 x *g* for 20 min to remove cellular debris. The supernatant was termed the cell-free extract and used for DMAT synthetase assay.

The crude enzyme extract from intact cells or cell free extract from protoplasts was placed in a dialysis bag and dialyzed against 10 mM Tris·HCl pH 8.0 buffer containing 20 mM calcium chloride and 20 mM 2-mercaptoethanol at 0° to 4°.

DMAT synthetase activity was measured by determination of the percent of conversion of [1-^{14}C] dimethylallylpyrophosphate ([1-^{14}C]-DMAPP) to dimethylallyltryptophan. Two methods were used for assaying DMAT synthetase. In the first method, enzyme fractions were incubated with appropriate amounts of L-tryptophan and [1-^{14}C]DMAPP in 10 mM Tris·HCl at pH 8.0, 20 mM calcium chloride and 20 mM 2-mercaptoethanol in a volume of 0.25 ml at 30° for 30 min. The reaction was stopped by chilling and addition of 50 μl of 2N HCl in 80% ethanol. The mixture was incubated for 15 min. Fifty microliters of this mixture was spotted on a piece of filter paper (Whatman No. 1, 3" x ¾"), which was dried under a hairdryer and steamed for 15 min to remove the volatile dimethylallyl alcohol. The strip was dried and placed in 10 ml of Bray's solution for radioactivity measurement. A blank with no tryptophan was run in order to determine the background due to polymerization of dimethylallylpyrophosphate. In the second method, which was developed by R. Hyslop in our laboratories, incubations were done under the same conditions as in the paper assay. The reaction was stopped by chilling and addition of 50 μl of 2N hydrochloric acid in 80 ml ethanol. The mixture was incubated for 15 min and was extracted three times with 2 ml of ethyl ether to remove the dimethylallyl alcohol. The aqueous portion was made alkaline with saturated aqueous sodium bicarbonate and placed in 10 ml of Aquasol scintillation liquid for radioactivity measurement.

The lactic dehydrogenase assay utilized the method of Kornberg (12). The mycelium from a 5-day-old shake culture of the fungus was ground in a mortar with ice-cold acetone and, after being filtered, dried at room temperature. Five hundred milligrams of the mycelial powder was suspended in 15 ml of 0.1 M phosphate buffer (pH 7.4) and stirred at 0° to 4° for 2 hr. After the mixture was centrifuged at 18,000 x *g* for 20 min, the supernatant was dialyzed against 0.1 M phosphate buffer (pH 7.4) at 0° to 4° for 18 hr. The dialyzed preparation was incubated with a 2 mM concentration of elymoclavine succinate either at 22° or 33° for 30 min in each case; then the incubation mixture was chilled to 0° to stop the reaction before enzyme activity was measured.

Protein was estimated by the method of Lowry *et al.* (13) and, when interfering buffer constituents were present, by the method of Bramhall *et al.* (14).

CHEMICALS.—Elymoclavine was isolated from *Claviceps* species, strain SD 58 shake culture fermentations by methods previously reported (15). L-[methylene-^{14}C] tryptophan (52 m Ci/mmole) was purchased from Amersham Corp. and [1-^{14}C]DMAPP was prepared as reported earlier (5).

RESULTS

INHIBITION OF ALKALOID SYNTHESIS BY ELYMOCLAVINE IN INTACT CELLS.—Our initial studies involving the addition of varying concentrations of elymoclavine succinate to 5-day-old shake cultures of the fungus gave an indication that there is some inhibition of alkaloid synthesis in intact cells of the organism.

As observed in three separate experiments, in cultures with added elymoclavine the incorporation of L-tryptophan into alkaloids was approximately one-half the incorporation shown in the control cultures at 6 hr after the addition of tryptophan. However, at 12 hr after tryptophan was added to the cultures, the rate of alkaloid synthesis, as measured by tryptophan incorporation, was approximately the same in both experimental and control cultures. An example of a typical experiment

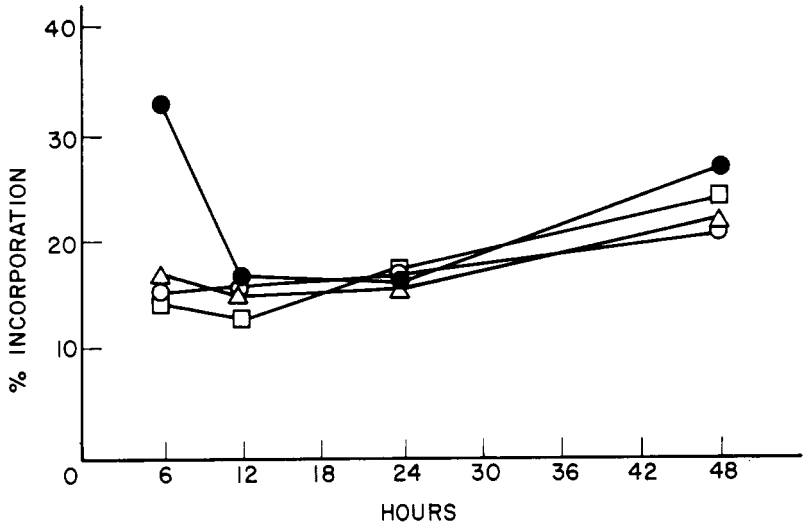

FIG. 1. Incorporation of L-tryptophan into ergot alkaloids by intact cells of *Claviceps* in the presence of varying concentrations of elymoclavine succinate added to 5-day-old mycelium growing in NL-406 shake cultures. The control culture (●) contained a 3 mM concentration of ammonium succinate and the concentrations of elymoclavine succinate were 1 mM (○), 2 mM (□), and 3 mM (△). The cultures were each incubated with 1 μCi of L-[methylene-^{14}C]tryptophan which was added 3 hr after the addition of the elymoclavine succinate and the sampling was timed from when the tryptophan was added.

is illustrated in fig. 1. A possible explanation for this result could be the synthesis of endogenous alkaloid by the cells of the control culture to a level which would produce the inhibition effect. In order to minimize the influence of endogenous alkaloid, intact cells of 5-day-old cultures were replaced into fresh medium containing elymoclavine succinate. As indicated by L-tryptophan incorporation into alkaloid, a partial inhibition of alkaloid synthesis resulted. The maximum inhibition occurred at 6 hr after the addition of tryptophan with an average of 44% incorporation in the controls and a 29% incorporation in the experimental culture (fig. 2).

Fig. 3 illustrates that when 11-day-old mycelium of a stationary culture was exposed to a 3 mM concentration of elymoclavine, there was at 24 hr more than a two-fold decrease in the incorporation of tryptophan into alkaloid when compared to the control. At six and one-half days after exposure to elymoclavine, there was essentially very little difference in alkaloid synthesis between the control cultures and the experimental cultures. Both sets of cultures showed a two-fold decrease in synthesis as compared to the maximum rate of synthesis at 24 hr after replacement. Measurements of alkaloid levels produced in control cultures indicate that at six and one-half days after replacement, the alkaloid concentration in the control cultures had reached an inhibitory level. Mycelium which had been exposed to a 3 mM concentration of elymoclavine for seven days was then exposed to fresh medium which did not contain elymoclavine. At 24 hr after this replacement, alkaloid synthesis was slightly higher in the control cultures

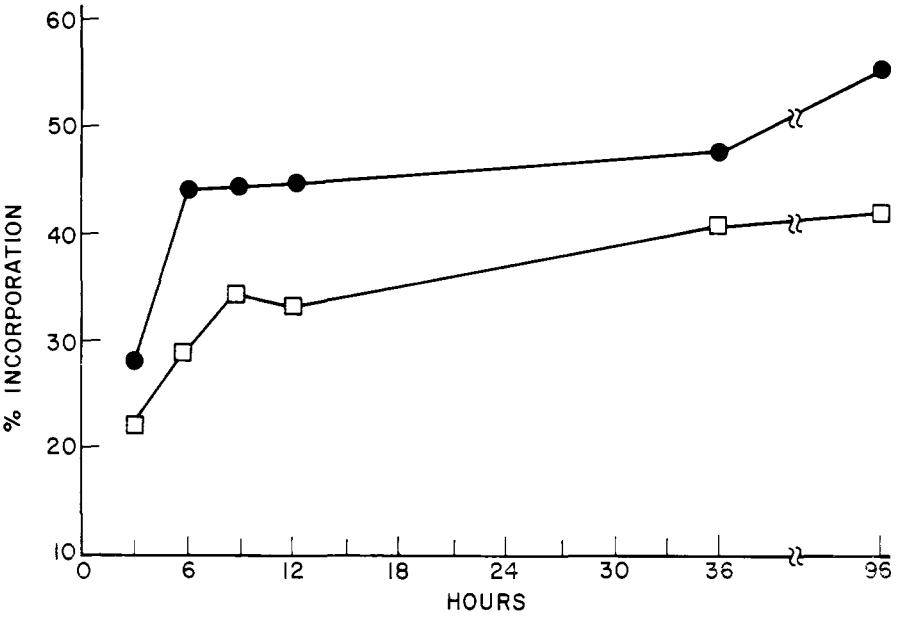

FIG. 2. Incorporation of L-tryptophan into ergot alkaloids by intact cells of *Claviceps* which were replaced after 5 days of growth in NL-406 shake cultures into NL-406 medium containing either 3 mM of potassium succinate as the control (●) or 3 mM elymoclavine succinate (□). The addition of radioactive tryptophan and sampling procedures were the same as described for fig. 1.

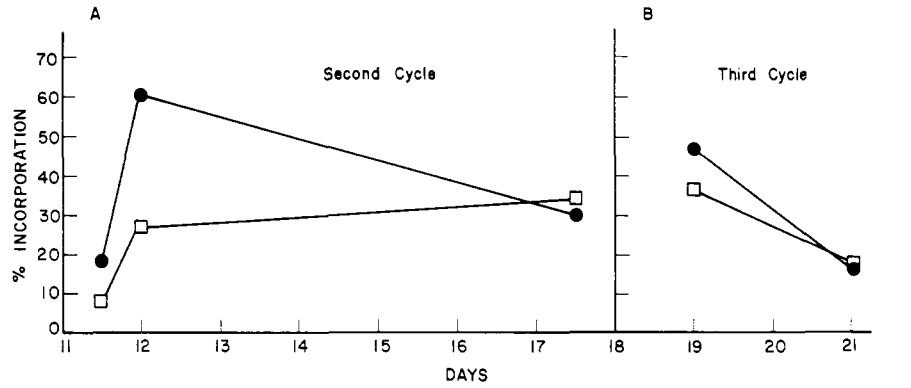

FIG. 3. Incorporation of L-tryptophan into ergot alkaloids by intact cells of *Claviceps* grown in stationary culture in which the medium was replaced as follows: (A) at 11 days with a medium containing either 3 mM potassium succinate as control (●) or 3 mM elymoclavine succinate (□). After replacing the medium, 0.5 μCi of L-[methylene-^{14}C]-tryptophan was added to both a control culture and an experimental culture at 0 hr, 12 hr, and 6 days, respectively, and harvested 12 hr later. (B) At 18 days after a 7 day exposure to a medium containing either 3 mM potassium succinate as control (●) or 3 mM elymoclavine succinate (□), the medium was replaced with fresh NL-406 medium free of potassium succinate and elymoclavine succinate. Radioactive L-tryptophan as in (A) was added at 12 hr and 36 hr, respectively, after replacing the media and harvested 12 hr later.

which had not previously been exposed to elymoclavine; however, after 3 days in the fresh medium, alkaloid synthesis in both control and experimental cultures decreased to approximately the same level (Fig. 3). Consistent with these results we found a three-fold increase to a possible inhibitory level of *de novo* alkaloid synthesized between 12 hr to 3 days after replacement.

An additional parameter of *in vivo* inhibition was investigated by measurement of DMAT synthetase activity in mycelia grown in stationary culture before and after exposure to a 3 mM concentration of elymoclavine. Fig. 4 illustrates that elymoclavine inhibits DMAT synthetase approximately three-fold as compared to the control after a 5-day exposure.

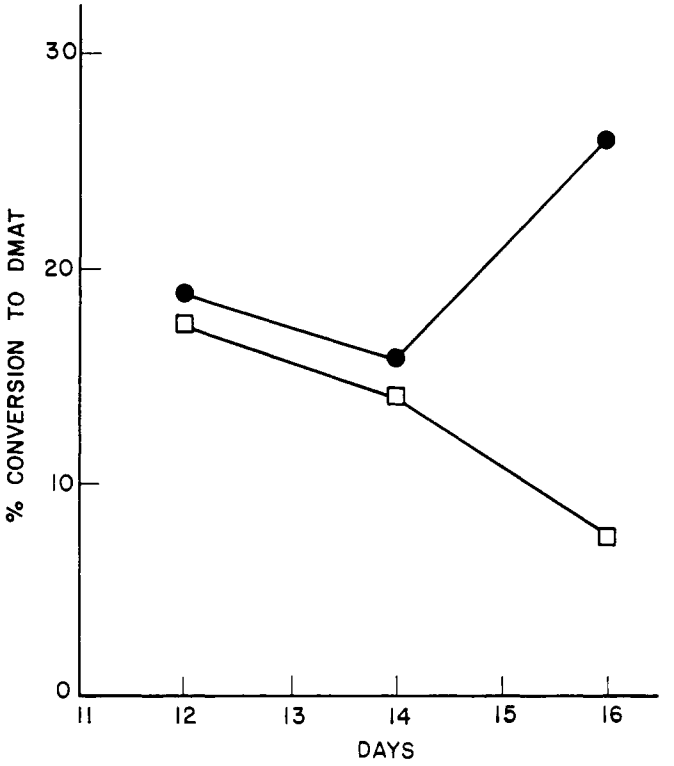

FIG. 4. DMAT synthetase activity in enzyme extracts, dialyzed for 4 hr, obtained from intact cells of *Claviceps* grown in stationary culture in which the medium was replaced at 11 days with a medium containing either 3 mM potassium succinate as control (●) or 3 mM elymoclavine succinate (□).

INHIBITION OF ALKALOID SYNTHESIS BY ELYMOCLAVINE IN PROTOPLASTS.—Protoplasts of *Claviceps* prepared by the method of Stahl *et al.* (9) provided an effective system for alkaloid inhibition studies. By microscopic examination of the protoplast suspensions, it was possible to monitor the success of protoplast formation. The viability of the protoplasts was determined by the regeneration of intact cells from the protoplast suspensions. These regenerated cells regained high alkaloid-producing ability and acquired the same culture characteristics

and morphology as the original cells from which the protoplasts were produced. Using ^{14}C-tryptophan and protoplasts suspended in 0.7 M KCl we were able to demonstrate alkaloid synthesis in this system. To measure elymoclavine inhibition of alkaloid synthesis the incorporation of L-tryptophan into alkaloid was compared to the control (fig. 5). Samples taken at 12 and 24 hours after the

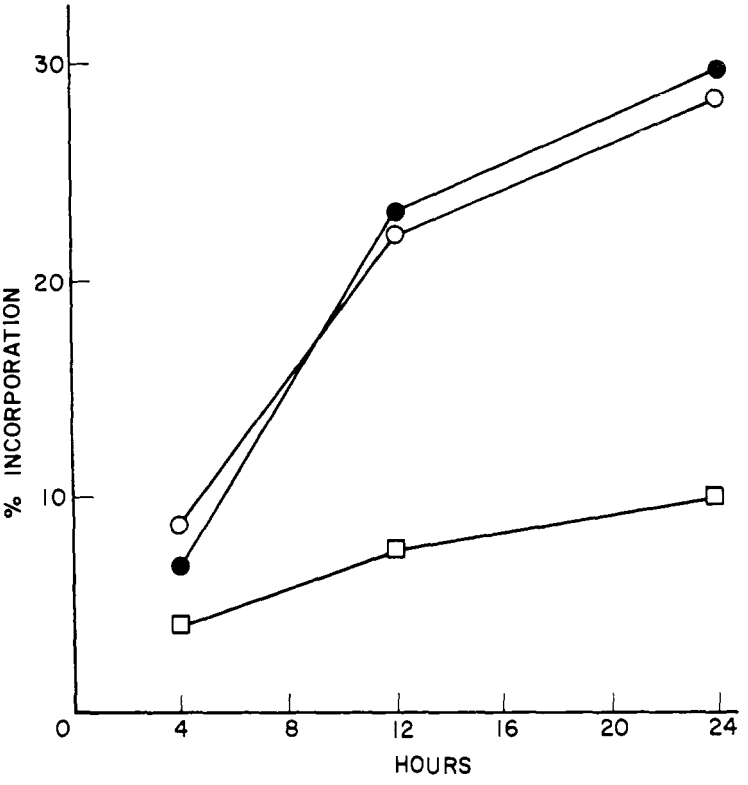

FIG. 5. Incorporation of L-tryptophan into ergot alkaloids by protoplasts of *Claviceps* prepared from 4-day-old mycelium. The incubations consisted of flasks containing protoplasts in 0.7 M KCl alone (●) and with 4 mM potassium succinate, (○) respectively, as controls and with 4 mM elymoclavine succinate (□). Each flask was sampled at 4, 12, and 24 hr after the addition of 2 μCi L-[methylene-^{14}C]-tryptophan.

addition of labeled tryptophan show, in both cases, a 3-fold decrease in alkaloid synthesis against the controls by protoplasts exposed to elymoclavine. Fig. 5 illustrates the results from a single experiment and is representative of similar results obtained in four independent experiments.

Samples were also analyzed for DMAT synthetase activity using protoplasts in which the rate of alkaloid synthesis was measured by tryptophan incorporation into alkaloid; at the same time, DMAT synthetase activity was measured in the protoplasts after a 20 hr. dialysis of the enzyme preparations, which effectively removes residual alkaloid. Fig. 6 illustrates the results of this experiment. Elymoclavine inhibits alkaloid synthesis by approximately 50 percent as indicated

by the rate of incorporation of tryptophan into alkaloid. Whereas, after removal of alkaloid from the enzyme preparations by dialysis, the elymoclavine treated protoplasts possess DMAT synthetase activity almost equal to that of the untreated

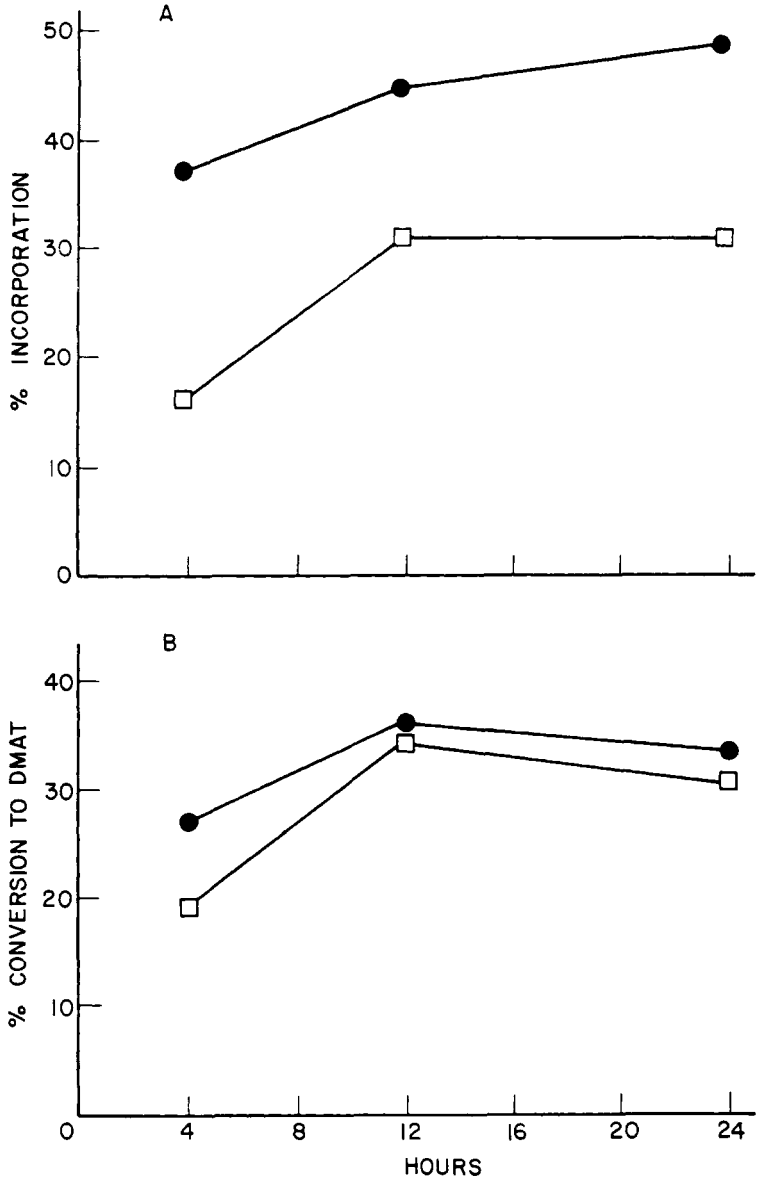

FIG. 6. The effect of removal of residual alkaloid from enzyme preparations of protoplasts. (A) Incorporation of L-tryptophan into ergot alkaloids by protoplasts of *Claviceps* using the same conditions as in fig. 5. Incubations consisted of controls with 4 mM potassium succinate (●) and experimental cultures with 4 mM elymoclavine succinate (□). (B) DMAT synthetase activity in enzyme extracts dialyzed for 20 hr to remove residual alkaloid. Incubations were the same as in (A).

protoplasts. These data provide strong evidence that end-product regulation of alkaloid synthesis in ergot involves feedback inhibition.

The specificity of the elymoclavine inhibitory effect on enzyme activity in *Claviceps* was tested by an *in vitro* assay of lactic dehydrogenase (EC 1.1.1.27) activity after incubation with a 2 mM concentration of elymoclavine. The enzyme activities in the control and in 22° and 33° incubations with elymoclavine are of the same order, indicating that elymoclavine does not unspecifically affect enzymes (fig. 7).

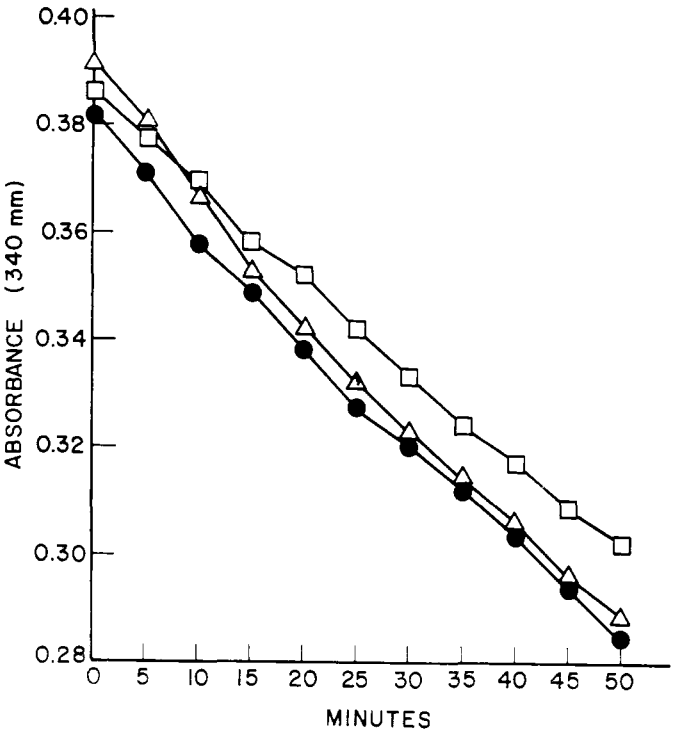

FIG. 7. The *in vitro* effect of elymoclavine on lactic dehydrogenase activity in dialyzed enzyme extracts prepared from 5-day-old mycelium of *Claviceps*. Incubations were with 2 mM elymoclavine succinate at 22 C (△) and 33 C (□) and control at 22 C (●).

DISCUSSION

The metabolic control which the end product of a biosynthetic pathway exerts over its own rate of formation by inhibiting the first pathway-specific enzyme is a well-known phenomenon in primary metabolism (16, 17). In secondary metabolism, only a few examples are known and the evidence for feedback inhibition is usually derived from physiological observations, i.e., addition of end product to cultures of different stages of growth and/or production, respectively. In the experiments reported here, *in vivo* feedback inhibition has been demonstrated.

The several approaches that we used to demonstrate *in vivo* regulation of alkaloid synthesis by a pathway end product, namely elymoclavine, met with varying degrees of success. Although the addition, under normal fermentation conditions, of certain antibiotics such as chloramphenicol (18), penicillin (17),

cycloheximide (19), and aurodox (20) have suggested that the synthesis of these antibiotics is under regulatory control by the end product; in the case of ergot this type of experiment gave the most unsatisfactory result. A possible explanation for this is that a permeability barrier exists which prevents the uptake of elymoclavine into the cell. This view is supported by the fact that addition of varying concentrations of elymoclavine causes no significant differences in the rate of alkaloid synthesis. As we have explained previously in the results section, the rapid decrease of alkaloid synthesis between 6 and 12 hr. in the control cultures (fig. 1) could be caused by *de novo* formed alkaloid reaching an inhibitory level during this time.

Utilizing replacement culture techniques in both shake and stationary cultures (figs. 2, 3, and 4) provided somewhat more convincing evidence for feedback regulation in intact cells. This methodology delays the influence of *de novo* alkaloid synthesis in the control cultures for a longer period of time since alkaloid which is synthesized and excreted into the medium prior to replacement has been removed. The net effect of this is to decrease endogenous alkaloid concentrations due either to a change in concentration gradient so that endogenous alkaloid diffuses from the cell or to an active excretion mechanism which removes alkaloid from the cell.

We found that end-product regulation of alkaloid synthesis could be most effectively demonstrated with the use of protoplasts of *Claviceps*. The protoplasts were capable of *de novo* alkaloid synthesis as was evidenced by the incorporation of radioactively labeled L-tryptophan into the alkaloids. In addition, and importantly, it appears that the permeability barriers to elymoclavine which were seen in experiments using intact cells were at least partially eliminated. Use of a protoplast system also demonstrated inhibition by the end product in the case of actinomycin biosynthesis (21).

When the percent incorporation rates shown in fig. 5 are compared with those illustrated in fig. 6, it can be seen that the values obtained vary between the two experiments. It is important to mention that within the individual experiments the results obtained are consistent in every case, namely, that exposure of protoplasts to elymoclavine succinate depresses alkaloid synthesis as evidenced by a decrease in the incorporation of tryptophan into alkaloid as compared to the control.

We have found in our experience that the absolute values obtained in physiological studies on ergot are greatly influenced by such factors as the age of the inoculum and the number of times the organism had been transferred prior to the preparation of the inoculum for the experimental cultures; therefore, it is difficult to make comparisons of absolute values between separate experimental trials.

As mentioned in the introduction, *in vitro* inhibition by elymoclavine has been demonstrated for DMAT synthetase (2, 6) by Erge *et al.* (7) for chanoclavine cyclase, and by Mann and Floss (8) for anthranilate synthetase. These observations raise the question as to whether or not the inhibition of alkaloid synthesis and DMAT synthetase activity in protoplasts exposed to elymoclavine is due to a specific end-product regulation or due to a toxic effect of elymoclavine on the cytoplasm and/or protein synthesis machinery. One can argue that elymoclavine is not toxic since it does not inhibit *in vitro* lactic dehydrogenase (fig. 7). Another indication of the specificity of this effect is the finding by Maier and Gröger (22) that DMAT synthetase from *Claviceps purpurea* strain Pepty 695,

a producer of the peptide alkaloid mixture ergotoxine, is not inhibited by agroclavine or elymoclavine. Also our results show that intact cells which have regained the full alkaloid-producing ability and morphology of the parent strain can be regenerated from elymoclavine-treated protoplasts.

The mechanism for end-product regulation can be either feedback inhibition, repression, or both. The DMAT synthetase assay of extracts from intact cells in stationary culture shown in fig. 4 initially led us to believe that a repression mechanism was operating in addition to the feedback inhibition. In this case a 4 hr dialysis of enzyme extracts did not restore DMAT synthetase activity to the level which was observed in extracts from control cultures. However, we can attribute this result to the fact that a 4 hr dialysis was not sufficient to remove residual alkaloid below an inhibitory level. As shown in fig. 6, when the enzyme extracts of normal and elymoclavine-exposed protoplasts have been carefully dialyzed for 20 hr to remove residual alkaloid, there is no significant difference in DMAT synthetase activity between the normal and the treated protoplasts. Our data provide strong evidence that end-product regulation of alkaloid synthesis in ergot involves feedback inhibition. They suggest that repression of enzyme synthesis by the end product may be of lesser importance for the regulation of alkaloid synthesis in *Claviceps* strain SD 58.

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