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Physiological Studies on Ergot: Further Studies on the Induction of Alkaloid Synthesis by Tryptophan and Its Inhibition by Phosphate

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The failure of L-leucine to stimulate ergot alkaloid production in a synthetic medium indicates that the previously observed stimulation by tryptophan and tryptophan analogues does not merely represent a nutritional effect. Tryptophan, but not mevalonate or 5-methyltryptophan, is able to overcome the inhibition of alkaloid synthesis by high levels of inorganic phosphate. Therefore, high phosphate levels seem to limit the synthesis of tryptophan; they may, in addition, prevent induction of alkaloid synthesis by preventing accumulation of tryptophan. Experiments which indicate a 2- to 3-fold temporary increase of intracellular free tryptophan and a 20- to 25-fold increase of tryptophan synthetase activity during the transition period between growth and alkaloid production phase are in agreement with the previously postulated induction of alkaloid synthesis by tryptophan. The latter experiments also indicate 4- to 6-fold repression of this enzyme by tryptophan.

Observations indicating an induction of the biosynthesis of ergot alkaloids by the precursor tryptophan have been reported by Floss and Mothes (3), Bu'Lock and Barr (1), Vining (16), and Robbers and Floss (10). Physiological evidence supporting the occurrence of this induction phenomenon has been provided by the work of Bu'Lock and Barr (1), who found that continued protein synthesis is required to maintain alkaloid production. Heinstein et al. (5) isolated dimethylallyl pyrophosphate:tryptophan dimethylallyl transferase which catalyzes the first reaction in the biosynthetic pathway to the alkaloids. They found that the activity of this enzyme increased rapidly 1 day before the appearance of alkaloids in the culture medium. An increase in the endogenous tryptophan pool during or just prior to the start of the transition period between the growth phase and the alkaloid production phase could be the trigger that would start alkaloid synthesis. We have undertaken studies to determine the level of free tryptophan in the mycelium during the transition from growth phase to alkaloid production phase and, to gain insight into the regulation of the free tryptophan pool, to determine tryptophan synthetase activity in the presence and absence of exogenous tryptophan.

Mary et al. (7), de Waart and Taber (2), and Taber (13) noted that a high concentration of inorganic phosphate in saprophytic cultures of ergot inhibits alkaloid synthesis. It can be postulated that excess phosphate, required for the production of protein and nucleic acids, prolongs the growth phase of the organism. During growth, amino acids such as tryptophan are utilized predominantly in the synthesis of protein. With the termination of the growth phase, tryptophan becomes available to serve as an inducer of the alkaloid-synthesizing enzymes and as a precursor in the synthesis of alkaloids (18); prolongation of the growth phase results in the inhibition of alkaloid synthesis due to lack of tryptophan accumulation. It is possible that phosphate may exert its effect in other ways, for example, by limiting the synthesis of endogenous tryptophan or by preventing tryptophan from acting as an inducer of the alkaloid-synthesizing enzymes. By exploring the influence of tryptophan on the high phosphate effect, we hoped to clarify aspects of the induction phenomenon and also of the high phosphate effect.

MATERIALS AND METHODS

Organisms. *Claviceps* species, strain SD-58, was used and is deposited in the culture collection of the

Department of Medicinal Chemistry and Pharmacognosy, Purdue University. Strain SD-58 was originally isolated from sclerotia obtained from the host, *Pennisetum typhoideum* Rich. *Escherichia coli* strain YS-100 was obtained from R. L. Somerville, Department of Biochemistry, Purdue University.

General culture techniques for ergot. *Claviceps* species, strain SD-58, was grown in shake culture at 24 C in a medium containing the following ingredients: mannitol, 50 g; sucrose, 50 g; succinic acid, 5.4 g; yeast extract, (Difco), 3.0 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.0044 g; boiled tap water, 1 liter; *p*H adjusted to 5.4 with NH_4OH. This medium is designated NL-406. Unless otherwise stated, the inoculum was prepared by replacing the mycelium from 100-ml shake cultures into 150 ml of sterile distilled water and resuspending the mycelium.

Influence of phosphate concentration on alkaloid production. Two-milliliter quantities were used to inoculate the experimental flasks. They comprised two groups, one consisting of NL-406 medium with an additional 1.0 g of KH_2PO_4 per liter and the other with no additional phosphate. Within each group, four series of flasks were prepared, each containing 25 ml of medium. The series contained the following additives: (i) 2 mM L-tryptophan, (ii) 1 mM DL-mevalonic acid, (iii) 2 mM L-tryptophan plus 1 mM DL-mevalonic acid, and (iv) control flasks with no additives. Solutions of the additives were introduced aseptically into the culture flasks by use of membrane filters (Millipore Corp.). One flask of each series was harvested each day for use in determining alkaloid production and mycelial dry weight.

Induction of alkaloid production. In the preparation of the inoculum, the mycelium was first homogenized in a blendor (Waring) before it was replaced. Three-milliliter quantities were used to inoculate each of the experimental flasks. All experimental flasks contained 25 ml of a basic medium of NL-406 plus 1.0 g of KH_2PO_4 per liter; however, yeast extract was omitted. Series of flasks were provided with the following additives: (i) 4 mM DL-tryptophan, (ii) 2 mM L-tryptophan, (iii) 2 mM D-tryptophan, (iv) 4 mM 5-methyltryptophan, and (v) control flasks with no additives. The additives were incorporated into the medium before autoclaving. Duplicate flasks of each series were harvested each day.

For a second induction experiment, 2-ml quantities were used for the inoculum. All experimental flasks contained 100 ml of a basic medium of NL-406 without the yeast extract. This medium also served as the control flask medium. Additions to the basic medium were as follows: (i) 1.0 g of KH_2PO_4 per liter, (ii) 1.0 g of KH_2PO_4 per liter plus 4 mM DL-5-methyltryptophan, (iii) 4 mM DL-tryptophan, (iv) 4 mM DL-5-methyltryptophan, and (v) 2 mM DL-leucine. By using sterile pipets, 10-ml amounts were removed from duplicate flasks each day and subjected to quantitative procedures.

Quantitation of alkaloids in micrograms per milligram of mycelial dry weight. The following procedures were employed on the experimental cultures to determine the amount of alkaloid in micrograms per milligram of mycelial dry weight. For dry-weight determination, the mycelium was removed from the culture filtrate by vacuum filtration and dried in a forced-air drying oven at 50 C for 24 hr. After measuring the total volume of the culture filtrate plus washings, a 2-ml amount was made alkaline by adding 0.2 ml of 10% ammonia solution and was extracted with 2 ml of $CHCl_3$. One milliliter of the $CHCl_3$ extract was evaporated to dryness with nitrogen, and 1 ml of 2% succinic acid solution was added. A colorimetric determination was performed by adding 2 ml of van Urk's reagent (12, 15), letting it stand for 20 min, and measuring the extinction at 580 nm. Van Urk's reagent contains 200 mg of *p*-dimethylaminobenzaldehyde and 0.15 ml of 10% $FeCl_3$ solution per 35 ml of distilled water and 65 ml of concentrated sulfuric acid.

Comparison of endogenous tryptophan levels with alkaloid production. Flasks containing 100 ml of NL-406 medium were inoculated with 1 ml of inoculum from a 7-day-old, 100-ml shake culture of strain SD-58. Every 24 hr, three flasks were harvested by removing the mycelium by vacuum filtration. The fresh mycelium was freeze-dried immediately, and an amount of the culture filtrate was frozen for eventual alkaloid determination. The dry mycelial samples were ground separately to a fine powder with a mortar and pestle. A 2-ml amount of boiling water was added to 100 mg of the powdered mycelium, and the resulting suspension was centrifuged at 20,000 $\times$ *g* for 15 min; the supernatant fluid, after passage through a membrane filter, was placed in a sterile culture tube.

For the tryptophan bioassay, a tryptophan-requiring mutant of *E. coli* was used. It was grown in 10 ml of nutrient broth for 17 hr at 37 C. The cells from the culture were freed from broth tryptophan by centrifuging the culture in a sterile tube, removing the supernatant fluid, washing with sterile water, centrifuging to remove washings, and suspending the cells in 10 ml of sterile water. Of the washed, resuspended cells, 0.2 ml was added to the assay medium along with 1 ml of the solution to be assayed. The assay medium consisted of 0.2 ml of Vogel/Bonner salt solution (17) and 9.7 ml of distilled water per culture tube. Vogel/Bonner salt solution was prepared as follows. In 670 ml of distilled water, 10 g of $MgSO_4 \cdot 7H_2O$, 100 g of citric acid $\cdot 1H_2O$, 500 g of anhydrous K_2HPO_4, and 175 g of $NaNH_4HPO_4 \cdot 4H_2O$ were dissolved successively with stirring at room temperature. This mixture was sterilized in the culture tube, and 0.1 ml of sterile 40% glucose solution was added. For the assay, the culture tube was incubated at 37 C for 17 hr. The extinction of the solution was measured at 550 nm, and the values obtained were compared with a standard curve.

Determination of free amino acid pool. Powdered, dried mycelium was extracted in the manner described for the endogenous trypophan assay. According to the procedure of Troll and Cannan (14), 0.5 ml of aqueous amino acid extract, 1 ml of KCN-pyridine solution, and 1 ml of 80% phenol solution

were added to each assay tube. After thorough mixing, the assay tube was placed in a boiling-water bath. When the mixture in the tube reached 98 C, 0.2 ml of ninhydrin solution was added, and the tube was heated for 5 min. Upon cooling with tap water, the volume was made up to 10 ml with 60% ethanol. The extinction was measured at 570 nm and compared with a standard curve determined with DL-leucine.

Determination of tryptophan synthetase activity. Two-milliliter amounts were used to inoculate two series of 100-ml cultures, one containing NL-406 medium and the other containing 40 mg of L-tryptophan per 100 ml of NL-406 medium. Cultures were harvested starting at 3 days of growth and every 24 hr thereafter. After the mycelial wet weight and the volume of culture filtrate were determined, a portion of mycelium was used for dry-weight determination, and the alkaloids were measured by using the culture filtrate. The remaining mycelial cells were disrupted by sonic treatment at 0 to 4 C for 3 min with glass beads and 0.1 M phosphate buffer (*p*H 7.8). The remaining homogenate was centrifuged at 1,000 × *g* for 10 min and at 25,000 × *g* for 20 min. After saturating the superantant fluid with $(NH_4)_2SO_4$ to a 90% concentration, it was centrifuged at 27,000 × *g*. The resulting precipitate was dissolved in 0.1 M phosphate buffer (*p*H 7.8) and dialyzed against buffer for 3 hr.

For the determination of tryptophan synthetase activity, 0.15 ml of the enzyme preparation was incubated at 37 C for 40 min with the following: 0.2 μmole of indole-2-^{14}C (1.5 × 10^3 dpm/nmole); 40 μmoles of DL-serine; 0.5 μmole of glutathione; 0.04 μmole of pyridoxal phosphate; and 25 μmoles of phosphate buffer (*p*H 7.8) in a total volume of 0.5 ml. After incubation, 0.1 ml of a 5 mM solution of L-tryptophan was added, and the mixture was heated for 5 min at 100 C. The solution was extracted with 1 ml of benzene 5 times, and 0.2 ml of the aqueous phase was chromatographed overnight (8 to 12 hr) on a paper strip in *n*-propanol-NH_4OH-water (6:3:1). The chromatogram was scanned for radioactivity, and the radioactive zone corresponding to tryptophan was cut out. The radioactivity was determined in a liquid scintillation counter, and on the basis of the specific activity of indole-2-^{14}C, the tryptophan synthetase activity in nanomoles per gram per hour was calculated as follows: disintegrations per minute of tryptophan × total volume of incubation mixture/volume of incubation mixture spotted × (1.5 × 10^3) × volume of enzyme preparation added. Multiply by total volume of enzyme solution/wet mycelial weight × incubation time.

RESULTS AND DISCUSSION

In our previous work (3, 10), we established that tryptophan and certain tryptophan analogues stimulate alkaloid production in the normal NL-406 medium and in fully synthetic medium lacking yeast extract; these findings led to the suggestion (3) that tryptophan acts as an inducer of the alkaloid-synthesizing enzymes. Growing the organism in a medium lacking yeast extract with ammonia as the only nitrogen source, while showing more clearly the influence of additives on alkaloid production, may have the disadvantage that the amino acid additives may be serving as nitrogen sources. If so, the increase in alkaloid production caused by these additives could be a nutritional effect rather than a specific enzyme induction. Previous work (10) with 5-methyltryptophan indicated that tryptophan analogues were not incorporated into protein. However, the possibility was not eliminated that the analogues are metabolized and parts of their molecules, e.g., the amino nitrogen, are utilized in protein synthesis. The utilization of L-leucine in an induction experiment has clarrified this point. Figure 1 shows that L-leucine, which would overcome the nitrogen starvation effect, does not stimulate alkaloid production, while both tryptophan and 5-methyltryptophan have a stimulating effect.

To further evaluate the induction phenomenon, we investigated the influence of tryptophan on the well-known inhibition of ergot

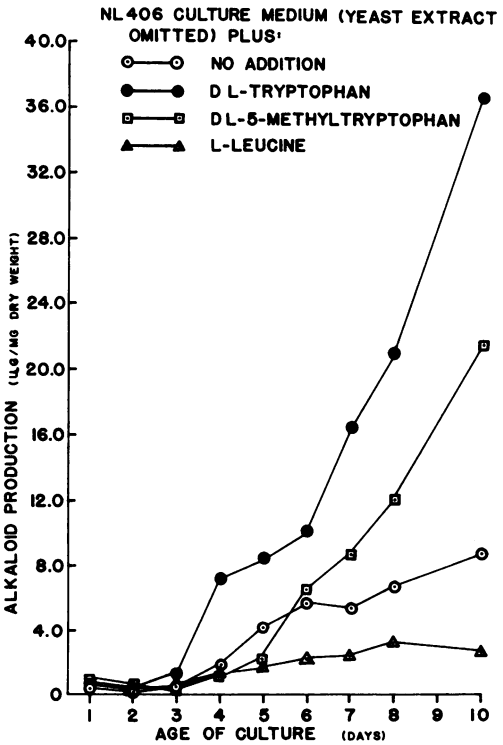

FIG. 1. *Comparison of the influence of DL-tryptophan, DL-5-methyltryptophan, and L-leucine on alkaloid production in Claviceps species, strain SD-58.*

alkaloid formation by high levels of inorganic phosphate. The ability of L-tryptophan to overcome the effect of high phosphate concentrations on alkaloid production is demonstrated in Fig. 2 and 3. Figure 3 illustrates that L-tryptophan stimulates an increase in alkaloid synthesis over the control cultures in the "normal" cultures. Earlier observations showed that tryptophan serves as a precursor in the synthesis of the alkaloids and acts as an inducer of the alkaloid-synthesizing enzymes. Mevalonic acid, on the other hand, exerts a slight negative influence on alkaloid production in the "normal" cultures. In the cultures containing high phosphate, tryptophan has partially overcome the phosphate effect on alkaloid formation, and mevalonic acid has no effect. This is evidence that the high phosphate effect on alkaloid production is mediated in some way through tryptophan, since tryptophan can overcome the effect.

As indicated earlier, there are a number of ways in which phosphate could act to bring about this inhibition of alkaloid synthesis. For example, high phosphate levels could directly block either the induction of the alkaloid-synthesizing enzymes or one of the steps in the

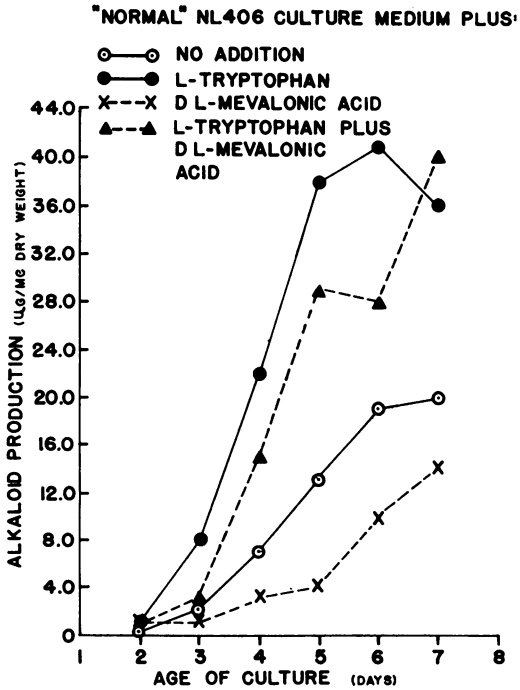

FIG. 2. *Comparison of the influence of* L-*tryptophan and* DL-*mevalonic acid on alkaloid production in Claviceps species, strain SD-58, in medium containing "normal" (0.1 g/liter) levels of* KH_2PO_4.

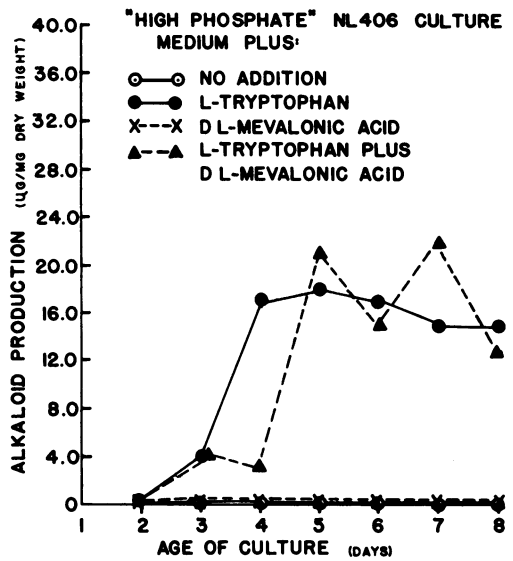

FIG. 3. *Comparison of the influence of* L-*tryptophan and* DL-*mevalonic acid on alkaloid production in Claviceps species, strain SD-58, in medium containing high (1.1 g/liter) levels of* KH_2PO_4.

biosynthetic pathway. This possibility is unlikely because of the ability of tryptophan to overcome the effect. More indirectly, high phosphate could influence alkaloid production by causing a depletion of the endogenous tryptophan pool or preventing tryptophan accumulation. This could be the result of an increase in or a prolongation of protein synthesis, or it could be due to the inhibition of enzymes involved in tryptophan biosynthesis, limiting the rate of tryptophan formation. DL-5-Methyltryptophan does not overcome the high phosphate effect (Fig. 4). It has been demonstrated (3, 10) that 5-methyltryptophan causes an increase in alkaloid production, presumably by induction, but cannot substitute for tryptophan as a precursor to the alkaloids. The discovery that the phosphate effect can be overcome by L-tryptophan, but not by 5-methyltryptophan, suggests that high concentrations of phosphate limit the formation of tryptophan, because mere addition of the inducer 5-methyltryptophan has no affect, but the addition of the substrate and inducer L-tryptophan restores alkaloid formation. High phosphate levels may prevent induction indirectly by preventing the accumulation of intracellular tryptophan, but this is not a necessary conclusion from these results. It would be reasonable to assume that 5-methyltryptophan can exert an inducer effect in cultures with the "normal" level of phosphate (*see* Fig. 1), because

tryptophan can be synthesized to serve as a precursor to the alkaloids, whereas in high phosphate cultures it cannot be synthesized in sufficient amounts.

Figure 4 also shows that D-tryptophan has some effect in restoring alkaloid synthesis in high-phosphate cultures. Since D-tryptophan can be converted to the L-isomer via the keto acid (4), this may also be a precursor effect.

If tryptophan-mediated induction of the alkaloid-synthesizing enzymes is a normally occurring process in the initiation of alkaloid formation, a number of predictions can be made which can be tested experimentally. Several of these have already been examined and verified. As indicated above, protein synthesis takes place during the alkaloid production phase (1, 6, 11), and alkaloid formation is dependent on continued protein synthesis (1). One of the enzymes involved in alkaloid synthesis, dimethylallyltryptophan synthetase, is essentially absent from growth-phase mycelium but appears during the transition period preceding alkaloid formation (5). Figure 5 shows the results of determination of endogenous mycelial tryptophan concentrations. It illustrates that the level of free tryptophan in the mycelium temporarily increases two- to threefold early during the transition from growth phase to alkaloid production phase. On the other hand, the free amino acid pool increases only by about 50% over the same period, indicating that increased tryptophan levels do not merely reflect an increase in the general amino acid pool. If tryptophan is involved in the induction of alkaloid synthesis, an increase of its concentration during the transition period, to exceed a triggering level, might be expected. These results are consistent with those shown in Fig. 6 where, during the transition period, the tryptophan synthetase activity increases 20- to 25-fold in both "normal" cultures and tryptophan-supplemented cultures. In this regard, this investigation compares with those of Rehacek et al. (8), and Rehacek and Malik (9), who found a sharp rise in tryptophan synthetase activity during the transition stage in a strain of *Claviceps paspali*.

Figure 6 also indicates that the level of tryptophan synthetase activity is controlled to some extent by exogenous tryptophan, since there is a four- to sixfold repression of the enzyme both during the growth phase and the alkaloid production phase when tryptophan is added to the cultures. However, the data of Fig. 6 strongly indicate that tryptophan synthetase is not solely controlled by end-product repression. A comparison of Fig. 6 and 5 shows

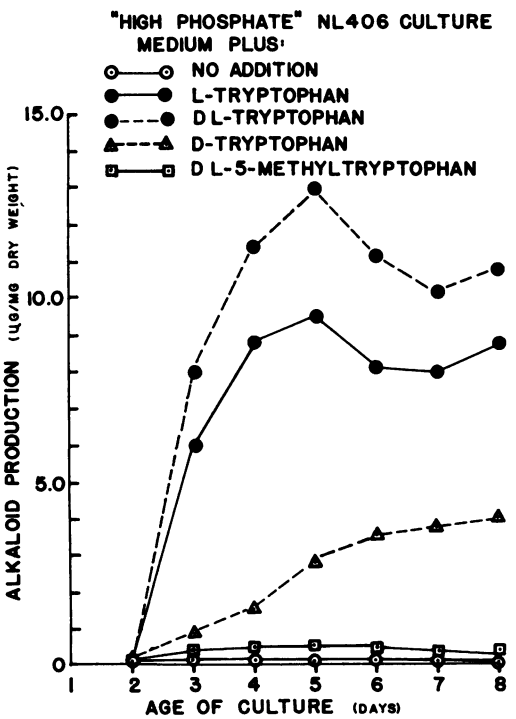

FIG. 4. *Comparison of the influence of* D L-*tryptophan,* L-*tryptophan,* D-*tryptophan, and* D L-*5-methyltryptophan on alkaloid production in Claviceps species, strain SD-58 in medium containing high levels of* KH_2PO_4 *(1.1 g/liter).*

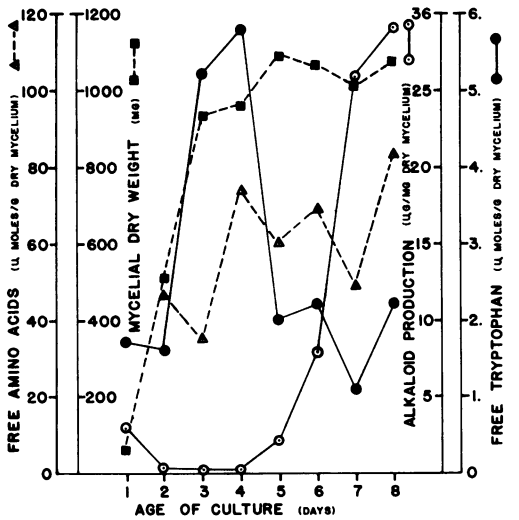

FIG. 5. *Comparison of mycelial growth, free endogenous tryptophan, free endogenous amino acids, and alkaloid production in Claviceps species, strain SD-58.*

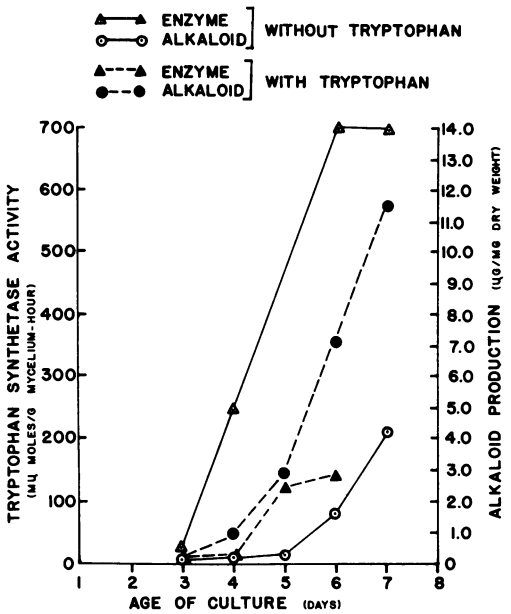

FIG. 6. *Comparison of alkaloid production and tryptophan synthetase levels in cultures of Claviceps species, strain SD-58, in normal and tryptophan-supplemented medium.*

that the changes in enzyme activity are not, in any simple and obvious way, related to changes in total free tryptophan levels in the mycelium. It is possible that the relationship is masked by the presence of more than one tryptophan pool in the cells. Earlier attempts to demonstrate the presence of more than one tryptophan pool in *Claviceps* strain SD-58 have, however, been unsuccessful (6). A particularly interesting finding in the experiments shown in Fig. 6 is the increase of tryptophan synthetase activity at about day 5 in the cultures supplemented with exogenous tryptophan. Measurements using analogous cultures from a different experiment have shown that the intracellular free tryptophan concentration at this time is several times higher than at any time in cultures not supplemented with tryptophan, and tryptophan synthetase should most likely be fully repressed. It is tempting to speculate that the observed increase in tryptophan synthetase is related to the induction of alkaloid synthesis. This phenomenon will be the subject of further investigations.

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