Correlation of Certain Alterations in Metabolic Activity with Alkaloid Production by Submerged Claviceps

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Alkaloid biosynthesis in Claviceps paspali MG-6 was favored by unbalanced growth. A positive correlation between the rate of protein turnover and alkaloid formation was noted. The pattern of the orthophosphate content in the mycelium resembled that in the ripening sclerotia of the parasitic strains. Alkaloids were revealed as potentially effective in energy metabolism. Reduced adenosine triphosphate (ATP) utilization and an increase in the ATP pool were found to be favorable for alkaloid production. Acetyl-coenzyme A carboxylase activity and the level of cell lipids were directly related to the intensity of alkaloid biosynthesis. An inverse relationship was observed between the activities of the tricarboxylic acid and glyoxylate cycles and the rate of alkaloid formation.

Although the production of ergot alkaloids by saprophytic strains of Claviceps has been a matter of increasing interest, additional data are needed on the physiology of their formation. In continuation of our previous work (26-29), we have investigated some cellular events accompanying alkaloid formation at various stages of submerged cultures of C. paspali and C. purpurea grown in a nutritionally poor medium. The purpose of this investigation was to gain information which will lead to a better understanding of the physiology of alkaloid biosynthesis.

MATERIALS AND METHODS

Organisms and culture conditions. C. paspali (Stevens and Hall), strain MG-6, and C. purpurea (Fr.) Tul, strain Pla-4, were used in this work. The synthetic medium was patterned after that described by Kobel, Schreier, and Rutschman (14), and had the following composition (g/liter): D-sorbitol, 50.0; succinic acid, 36; K2HPO4, 2.0; MgSO4·7H2O, 0.01; the pH was adjusted to 6.0 with 1 N NH4OH. Fermentation experiments were carried out in 300-ml Erlenmeyer flasks containing 100 ml of medium. The flasks were incubated at 24 ± 1°C on a rotary shaker operating at 240 rev/min with a 5.5-cm stroke. Each shaken flask was inoculated with washed vegetative-cell inoculum equivalent to 7 to 10 mg of dry weight and blended for 15 sec. The inoculum was obtained from 5-day-old shaken cultures grown in 4.5% malt extract broth (Difco) in distilled water. The experimental design consisted of growing the cultures in a rich medium which did not favor alkaloid production and subsequently transferring them into a nutritionally poor medium in which alkaloids were formed. The mycelium from the latter medium was collected at various time intervals and analyzed as indicated.

Analytical methods. Protein was determined by the method of Lowry et al. (19), and nucleic acids were determined by the methods of Schneider (30) and Keen and Williams (13). For determination of lipids, the washed mycelium was precipitated with 5% trichloroacetic acid at 0°C for 60 min and extracted with ethanol and a mixture of ethanol-ether (1:3). The extracts were combined and evaporated in vacuo, and the lipid residue was weighed. Orthophosphate was determined by the method of Cole and Ross (5), pyruvic acid by the procedure of Boning (2), and ergot alkaloids as described earlier (27).

Extraction and assay of ATP. Samples (10 ml) were removed from the growing culture and centrifuged at 3,000 × g for 30 sec. The mycelium was boiled in 10 ml of 0.1 M phthalate buffer (pH 7.1) for 10 min (12, 32) and then centrifuged. The adenosine triphosphate (ATP) concentration in the supernatant fluid was determined by the luciferase method (20). Firefly lanterns, i.e., desiccated tails of Photinus pyralis (Sigma Chemical Co., St. Louis, Mo.), were the source of luciferin and luciferase. The reaction mixture was prepared by crushing 120 mg of fireflies and extracting them three times with 1 ml of distilled water. The extract was centrifuged, 0.3 ml of 4% phosphate gel (pH 8.0) was added, and the precipitate was removed by centrifugation. The reaction mixture contained 0.2 ml of 0.1 M MgSO4, 0.2 ml of 0.1 M Na2HAsO4 (pH 7.4), 0.3 ml of extract, 0.2 μg of ATP/0.2 ml of water or 0.1 to 0.3 ml of sample
tested, and 0.05 M glycine (pH 7.4) to bring the volume to 3 ml. The shaken mixture was assayed immediately in the Photomultiplier EMI 9635 QB.

**Protein synthesis.** The rate of protein synthesis was indicated by 14C-leucine incorporation into the trichloroacetic acid-insoluble fraction of mycelium of various ages. Samples (50 mg) of chilled mycelium were blended with 10 ml of supernatant fluid in a Waring Blendor at low speed for 30 sec. Subsequently, 5 ml of the blended mycelium was dispensed in a 25-ml conical flask containing 5.0 µCi of uniformly labeled 14C-L-leucine and 2.5 mM DL-leucine. The incubations were carried out on a Dubnoff metabolic incubator at 24 C for 60 min. Incorporation of leucine was stopped by adding 30 ml of cold 10% trichloroacetic acid to the samples. The trichloroacetic acid-treated samples were filtered through membrane filters (Synpor 5, diameter 24 mm, pore size 0.6 µm; Synthesia Ulbrichs) and washed with 3 ml of 100 µM DL-leucine and 1 ml of water. The layers of trichloroacetic acid-insoluble mycelial fractions were dried under an infrared lamp and placed in vials with 5 ml of scintillation fluid composed of 4.0 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-di-2-(5-phenyloxazolyl)benzene (POP0) in 1 liter of toluene. The radioactivity measurements were made in a liquid scintillation counter (Mark I, Nuclear-Chicago Corp., Des Plaines, Ill.). The values were expressed as counts per minute.

**Cell extracts for enzyme assays.** Crude cell extracts were prepared by disrupting the washed mycelium in a French pressure cell. The broken cells were extracted with an appropriate buffer. The resulting suspension was fractionated at 20,000 X g in a refrigerated centrifuge for 30 min, and the supernatant fluid was used for enzyme assay.

**Determination of enzyme activities.** The adenosine triphosphatase (EC 3.6.1.3) activity was measured by determining the amount of orthophosphate liberated from the substrate. A 1 ml reaction mixture, containing 0.3 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5), 0.3 µM of 10^-2 M ATP, 0.1 to 0.2 ml of cell-free extract (1.5 to 2.5 mg of protein/ml), and distilled water to a volume of 1 ml, was incubated for 20 min at 37 C. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid, and samples of clear supernatant fluid were analyzed for orthophosphate (5).

Adenylate kinase (EC 2.7.4.3) activity was measured by an ultraviolet test (Biochemica Test Combinations, UV-Method TC-I, catalog no. 15379 TAAC, Boehringer u. Soehne, 1969). A 1 ml reaction mixture, containing 0.1 ml of 0.1 M Tris-hydrochloride buffer (pH 7.5), 1 µMole of adenosine diphosphate (ADP), 0.1 ml of cell-free extract (1.5 to 2.5 mg of protein/ml), and distilled water to 100 ml, was incubated for 15 min at 20 C. After denaturation of enzyme activity by boiling the reaction mixture for 10 min, 0.2 ml of clear supernatant fluid was added to the reaction mixture containing 2.5 ml of 0.1 M triethylamine buffer (pH 7.6), 4 mmoles of MgSO4, 6 mmoles of 3-phosphoglycerate, 12 mmoles of reduced nicotinamide adenine dinucleotide (NADH), and 0.05 ml of standard enzyme preparation (1 mg of 3-phosphoglycerate kinase/ml plus 4 mg of nicotinamide adenine dinucleotide phosphate-glyceraldehyde-3-phosphate dehydrogenase/ml). The decrease in NADH was measured at 340 nm in 30-sec intervals for 5 min at 44 C.

Acetyl-coenzyme A (acetyl-CoA) carboxylase (EC 6.4.1.2) activity was determined by measuring the rate of H214CO3 incorporation into acetyl-CoA (23). Counting was done in Bray's solution containing 60 g of naphthalene, 4 g of PPO, 0.2 g of POPOP, 100 ml of methanol, 20 ml of ethylene glycol, and p-dioxane to a final volume of 1,000 ml.

Published methods were used for the determination of citrate synthase (EC 4.1.3.7; 31), malate synthase (EC 4.1.3.2; 8), isocitrate lyase (EC 4.1.3.1; 21), peptidases (10, 11), and proteases (24).

**RESULTS**

Association of alkaloid production with different growth phases. As shown in Fig. 1, the growth of strain MG-6 was slower than that of Pla-4. In the former strain, the biomass and alkaloid syntheses proceeded simultaneously. Alkaloids appeared in the medium well before significant release of nucleic acids into the culture supernatant fluid (Table 1), i.e., before extensive autolysis of the cells took place. The lysogenic acid methylcarbinolamide was a predominant component of the alkaloid mixture extracted from the culture medium. The alkaloid content in washed mycelium was negligible. During the alkaloid production phase, cell multiplication slowed down and the amount of mycelial protein

![Fig. 1. Content of mycelial proteins and lipids during alkaloid formation by C. paspali MG-6 and C. purpurea Pla-4. DW = dry weight; ALK = alkaloids; LI = lipids; PR = proteins.](image-url)
TABLE 1. Cell autolysis in submerged Claviceps cultures

| Time (days) | C. paspali MG-6 | C. purpurea Pla-4 |
|-------------|-----------------|-------------------|
|             | DNA* | RNA* | DNA | RNA |
| 0-7         | 0    | 0    | 0   | 0   |
| 8           | 0    | 0    | 0.9 | 0.02|
| 9           | 0.9  | 0.04 | 1.9 | 0.04|
| 10          | 1.2  | 0.05 | 4.3 | 0.09|

* Milligrams per liter of culture supernatant fluid.

TABLE 2. Distribution of nonprotein (A) and protein (B) components in mycelium of submerged Claviceps cultures

| Time (days) | C. paspali MG-6 | C. purpurea Pla-4 |
|-------------|-----------------|-------------------|
|             | A    | B   | A/B | A    | B   | A/B |
| 3           | 4.9  | 0.1 | 49  | 12   | 0.06| 198 |
| 5           | 15   | 0.3 | 52  | 25   | 0.3 | 85  |
| 7           | 35   | 0.7 | 50  | 14   | 0.6 | 22  |
| 8           | 51   | 1.0 | 46  | 13   | 0.2 | 65  |
| 9           | 43   | 2.0 | 20  | 13   | 0.2 | 65  |

* Milligrams per 10 ml of medium.

was essentially constant. From the data of Fig. 1, it appears that strain MG-6 accumulated lipids throughout the fermentation and that the higher dry weight was partially ascribable to lipid accumulation. The strain did not show any evident competition between lipid formation and alkaloid production (Fig. 1). The ratio between nonprotein and protein components of the mycelium was almost constant during alkaloid formation (Table 2).

In C. purpurea Pla-4, the alkaloid content increased after growth had ceased (Fig. 1) and autolysis had taken place (Table 1). A lag period was evident between the end of the growth phase and the onset of alkaloid production. The alkaloid mixture was comprised of ergine, ergometrine, ergometrinine, and isoseticlavine. The increasing alkaloid formation was inversely proportional to the amount of mycelial protein and was accompanied by a relatively constant level of mycelial lipids (Fig. 1). As indicated in Table 2, the ratio between nonprotein and protein components of the mycelium varied during the fermentation. In the early part of fermentation, this ratio was high and decreased to a level resembling that observed in strain MG-6 during alkaloid formation. The high nonprotein to protein ratio did not appear to favor alkaloid formation.

An analysis of whole cells revealed that the percentage of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was greater in strain MG-6 than in Pla-4. The DNA content of both strains was almost constant, whereas the amount of total RNA was variable during the fermentation (Fig. 2). The content of soluble RNA was very low in the early part of fermentation. The soluble RNA curve coincided with the curves of both total RNA and culture growth rate (Fig. 1 and 2). The insoluble RNA increased as the alkaloid production was enhanced. The decrease in protein synthesis (Fig. 3) coincided with the cessation of RNA synthesis in strain Pla-4 (Fig. 2).

A rapid "saturation" of the mycelium with orthophosphate occurred in the early growth phase of MG-6 (Fig. 4) and was three times higher than in Pla-4. After being temporarily constant, the amount of orthophosphate began to fall during the phase of prolonged slow growth and increasing alkaloid formation (Fig. 1 and 2). In strain Pla-4, orthophosphate accumulation proceeded slowly and rose only in the phase of

Fig. 2. Content of DNA and RNA in submerged mycelium of Claviceps strains MG-6 and Pla-4. RNA = total RNA; RNA\(_s\) = soluble RNA; RNA\(_i\) = insoluble RNA. Arrow indicates the time of maximal growth.
decreasing growth rate and enhanced alkaloid production. In both strains, 0.8 to 1.1 μmoles of mycelial orthophosphate/mg of protein was found during increasing alkaloid formation.

Synthesis and breakdown of cell proteins during alkaloid biosynthesis. Differences in the rate of protein synthesis by both strains were noted (Fig. 3). The fluctuation in protein synthesis in strain MG-6 did not influence the almost constant level of the mycelial protein (Fig. 1 and 3). On the other hand, the curve of protein synthesis intensity in strain Pla-4 was almost identical with that of mycelial protein. In both strains, the decrease in protein synthesis corresponded to the relative increase in alkaloid formation.

In view of these results, it was of interest to ascertain the levels of proteases produced by both cultures. Figure 3 shows that proteins in both strains were broken down intracellularly in multiplying cells. Intracellular proteases in strain MG-6 reached a maximum in the early log phase and then declined. Some proteases were truly extracellular and were liberated by continuous secretion and not by cell lysis. This secretion occurred in parallel with growth (Fig. 1 and 3), ceased at stationary phase, and declined during

**Fig. 3.** Alteration in rate of protein synthesis and activities of proteases and peptidases during submerged cultivation of Claviceps strains. 14C-LEU = protein synthesis intensity; IPR = intracellular proteases; EPR = extracellular proteases; IPE = intracellular peptidases; EPE = extracellular peptidases.

**Fig. 4.** Changes in amount of orthophosphate (Pi) during alkaloid production.

**Fig. 5.** ATP pool level and activities of adenine triphosphatase and adenylate kinase in submerged Claviceps cultures. AK = adenylate kinase activity.
rapid autolysis. There was a correlation between the secretory process and total protein synthesis. Protease activities of strain Pla-4 exceeded those of strain MG-6.

As distinct from proteases, peptidases manifested their activities only inside the cells of both strains (Fig. 3). The relative rate of peptidase induction occurred later than that of proteases, i.e., the pattern of induction was sequential.

**ATP pool at various culture stages.** Intracellular ATP was examined because of its role as the primary energy mediator and metabolic regulator. As shown in Fig. 5, the amount of ATP per mycelial mass (i.e., the ATP pool) was changing during the whole fermentation period.

The ATP pool in strain MG-6 increased at a higher rate than the culture growth rate during the exponential phase (Fig. 1 and 5). The ATP reached a maximum at the 7th day and a second less distinct maximum at the 9th day. A marked ATP rise was accompanied by a decrease in the adenosine triphosphatase activity. Properties of this Mg\(^{2+}\)-activated adenosine triphosphatase are listed in Table 3 (28). As depicted in Fig. 1 and 5, the adenosine triphosphatase activity was reduced during slow culture growth, and the increase in the ATP and alkaloid formation was stimulated. The adenosine triphosphatase activity in cell-free extracts of strain MG-6 was inhibited by added ergometrine (Fig. 6).

The ATP pool in strain Pla-4 was in balance with the rate of culture growth (Fig. 1 and 5). As growth proceeded, the concentration of ATP fell, owing to its ready accessibility to adenosine triphosphatase. This pattern was characterized by a low rate of alkaloid synthesis.

When 1 μmole of ADP was incubated with the crude cell-free extracts, ATP activity was found in samples of both strains. Thus, the presence of adenylyl kinase activity catalyzing the nonoxidative formation of ATP was indicated. Although the shapes of adenylyl kinase curves of both strains were different (Fig. 5), a similarity of the curves was observed in the period of alkaloid formation. Ergometrine (10\(^{-4}\) and 10\(^{-3}\) M) did not influence the adenylyl kinase activity in the cell-free extracts.

**Changes in activities of certain enzymes during alkaloid fermentation.** Our previous work (26) has shown an elevated level of some products of acetate catabolism in the cell pool of *C. paspali* MG-6. In this paper, extracellular accumulation of pyruvic acid during alkaloid fermentation was observed in both strains of *Claviceps*, indicating that the oxidative decarboxylation of pyruvate to “active acetate” entering the tricarboxylic acid cycle was limiting (Fig. 7). Extracellular accumulation of pyruvic acid by both strains followed almost an identical pattern. In the presence of arsenite, the pyruvic acid accumulation was slightly increased whereas alkaloid synthesis intensity was not markedly affected. These results indicated that the highly active oxidative metabolism of carbohydrate (1) was not of primary importance in the alkaloid biosynthesis.

Citrate synthase activity—the key indicator of the tricarboxylic acid cycle—reached 52 nmoles of citrate per mg of protein per min in strain MG-6, a level which was only 20% of that in strain Pla-4 (Fig. 8). It remained fairly steady at this level during an intensive alkaloid formation. In contrast, the citrate synthase activity in strain Pla-4 rose dramatically within 3 to 7 days of incubation and reached levels three to five times higher than those in strain MG-6, but the alkaloid yields were insignificant.

Since the glyoxylate cycle is an alternative

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**Table 3. Properties of adenosine triphosphatase in subcellular preparations of submerged *Claviceps* cultures**

| Determination          | *C. paspali* MG-6 | *C. purpurea* Pla-4 |
|------------------------|-------------------|---------------------|
| Mg\(^{2+}\) activation | Present           | Present             |
| Ca\(^{2+}\) activation | Absent            | Absent              |
| ATP to Mg\(^{2+}\) ratio | 0.1 or 0.05       | 0.1 or 0.05         |
| K\(^{+}\) stimulation  | 105% at 0.2       | 62% at 0.2          |
| Digestion product     | m ADP             | m ADP               |
| Optimal pH            | 7.5               | 7.5                 |

* Increase in activity in addition to that caused by Mg\(^{2+}\).
pathway to the tricarboxylic acid cycle, we investigated its key enzymes, i.e., isocitrate lyase and malate synthase. Activity of the enzymes was not detected during alkaloid production in the mycelium of strain MG-6, but was found in Pla-4 (Fig. 8). Concomitant with the citrate synthase in the latter strain, stimulation of isocitrate lyase and malate synthase production was recorded. As soon as the alkaloid synthesis intensified, the activities of all three enzymes began to decline.

Since alkaloid synthesis is often discussed in relation to fatty acid synthesis, we investigated whether the differences in the rate of alkaloid formation were associated with acetyl-CoA carboxylase activity, which catalyzes the regulatory and committed step in fatty acid biosynthesis (4, 9). In both strains, the acetyl-CoA carboxylase was almost directly proportional to the amount of lipids in the mycelium (Fig. 1 and 8). In addition, a definite correlation between the acetyl-CoA carboxylase activity and level of cell lipids, and the intensity of alkaloid biosynthesis was found in strain MG-6.

DISCUSSION

Slow growth of Claviceps cultures was observed when the cultures in the stationary phase were transferred from a complex medium into a nutritionally poor medium. There was no net RNA or protein synthesis in the early part of fermentation, and phenotypic changes might have occurred in these "shifted-down" cultures. Different cell components were formed at different intensities. Nutritional conditions which favor maximal primary shunt metabolism in a given case are not expected to favor maximal secondary shunt metabolism (3, 33, 34). Our results support this hypothesis. The biosynthesis of alkaloids appeared to be favored by unbalanced growth of the cultures. This holds true only for strain MG-6, as in strain Pla-4 alkaloids were formed only at the end of the incubation period. At this point, the key role of protein turnover in the synthesis of new enzymes in cultures grown in nutritionally poor media is of importance (22, 35). In light of this fact, and according to our data which demonstrated the occurrence of net protein synthesis at a constantly decreasing rate and the almost invariable level of cell protein, as well as the low protease activities in the "shifted-down" strain MG-6, we suggest a positive association between the rate of protein turnover and alkaloid formation.

Data already exist in the literature (7, 16) which are in agreement with our results, namely, that the ergot alkaloids are formed when the phosphate for abundant growth and synthesis of phosphorylated compounds is limiting. High ortho-

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**Fig. 7.** Extracellular accumulation of pyruvic acid during alkaloid fermentation. + As = 3-day-old cultures were treated with sodium arsenite (0.1 mM).

**Fig. 8.** Activities of certain enzymes in submerged Claviceps cultures. ACC = acetyl-CoA carboxylase; CS = citrate synthase; ICL = isocitrate lyase; MS = malate synthase.
phosphate content in the mycelium and a correlation between a pronounced decrease of orthophosphate level and enhanced alkaloid production observed in our experiments with saprophytic cultures resembled the pattern in ripening sclerotia of the parasitic strain of *C. purpurea* (16, 17).

The slow growth was probably a rate-limiting step in biosynthesis. The evidence that both the increase in ATP pool and reduced ATP utilization were favorable for alkaloid production leads to the suggestion that energy production was not limiting in alkaloid biosynthesis. The inhibition of the adenosine triphosphatase activity by added ergometrine is of particular interest, since it indicates for the first time that ergot alkaloid may be potentially effective in energy metabolism.

In strain MG-6, an inverse relationship was found between ATP and adenosine monophosphate (AMP). In the presence of low AMP concentrations, fatty acids and alkaloid biosynthesis competed more strongly for acetyl-CoA, whereas the tricarboxylic acid cycle, because of the presence of high ATP, competed more weakly (Fig. 9). This suggestion is supported by our previous data (26) in which an inverse relationship was shown between alkaloid formation intensity and cell-pool level of citric, succinic, fumaric, and oxalacetic acids. It was of interest that the two unique enzymes of the glyoxylate cycle, i.e., isocitrate lyase and malate synthase, have been shown to be entirely (in MG-6) or partially (in Pla-4) inactive during alkaloid formation, which further supports the notion that an inverse proportionality between the rate of alkaloid formation and the activities of the tricarboxylic and glyoxylate cycles exists.

Although the glyoxylate cycle has a number of reactions in common with the tricarboxylic acid cycle, there is mounting evidence that in eucaryotes the two cycles are physically separated in the cell. It has been shown (6, 15, 25) that most, if not all, of the glyoxylate cycle enzymes are in organelles called glyoxysomes, whereas the tricarboxylic acid cycle enzymes are in mitochondria. It thus might be deduced that mitochondria and glyoxysomes may participate in regulation of alkaloid biosynthesis.

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