# THE RELATIONSHIP BETWEEN INTENSITY OF OXIDATIVE METABOLISM AND PREDOMINANCE OF AGROCLAVINE OR ELYMOCLAVINE IN SUBMERGED *CLAVICEPS PURPUREA* CULTURES

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ABSTRACT.—Submerged cultures of *Claviceps purpurea* strain 129 and its mutants producing a mixture of chanoclavine, agroclavine, and elymoclavine are characterized with respect to the relationship between culture development, some enzyme activities, and alkaloid production. The cultures with dominant elymoclavine yield possess an intensive oxidative metabolism, efficient supply of glycolytic intermediates to the Krebs cycle, and a higher activity of the hexose monophosphate pathway. The oxidative metabolism and a sufficient NADPH supply is supposed to be involved in hydroxylation of agroclavine to elymoclavine.

Submerged cultures of *Claviceps purpurea* strain 129 and its mutants produce a mixture of chanoclavine, agroclavine, and elymoclavine. Agroclavine is a dominant component of the mixture (80–90%) in fermentation culture of the parent strain and high production mutants. On the other hand, vegetative inocula of both the strain 129 and high production mutants, and fermentation culture of a low-producing mutant 244 (1) contain predominately elymoclavine (60–80%). In this work the oxidative metabolism of the above-mentioned cultures was studied with respect to the formation of elymoclavine, *i.e.*, a hydroxylated derivative of agroclavine.

## MATERIAL AND METHODS

STRAIN AND CULTIVATION CONDITIONS.—*Claviceps purpurea* strain 129 originated from the collection of microorganisms of the Institute of Microbiology CAS. Its mutants were obtained after mutagenesis with uv light (2). The strains were cultivated under submerged conditions for 10 days in an inoculation medium T 1 (sucrose 100 g, L-asparagine 10 g, L-cystein HCl 0.1 g, yeast extract 0.1 g, $Ca(NO_3)_2 \cdot 4\,H_2O$ 1 g, $KH_2PO_4$ 0.25 g, $MgSO_4 \cdot 7\,H_2O$ 0.03 g, $ZnSO_4 \cdot 7\,H_2O$ 0.02 g, distilled water to make 1 liter, pH 5.2 with NaOH) which was inoculated with spore suspension. Spores were washed from the surface of the slant T2(3) with medium T I by means of a glass rod. The final spore count was about $2.10^6$ per flask. All spore counts were performed with a Bürker chamber for counting of blood cells. The fermentation medium CS 2 (sucrose 100 g, citric acid 16.8 g, $(NH_4)_2$-$SO_4$10 g, $CaCl_2$ 1.1 g, $KH_2PO_4$ 0.25 g, $MgSO_4 \cdot 7\,H_2O$ 0.25 g, KCl 0.12 g, $FeSO_4 \cdot 7\,H_2O$ 0.02 g, $ZnSO_4 \cdot 7\,H_2O$ 0.015 g, distilled water to make 1 liter, pH 5.2 with NaOH) was inoculated with 4 ml of the vegetative inoculum. Cultivations were done in the dark at 24±1° on a rotary shaker (4 Hz, excentricity 5.4 cm) in Erlenmeyer flasks (300 ml) containing 60 ml of the appropriate medium.

PREPARATION OF THE CELL-FREE EXTRACTS AND ENZYME ASSAYS.—In the course of cultivation, parallel cultures were harvested and pooled to obtain about 10 grams of mycelial wet weight. The mycelium was washed twice with distilled water and frozen at −20°. The stored samples were disintegrated in an X-press (Biotec, Sweden), suspended in 0.1 M Tris-HCl buffer pH 7.5, and centrifuged 30 min at 15 000 xg. 6-Phosphogluconate dehydrogenase (4) and glucose-6-phosphate dehydrogenase (5) were immediately assayed in the supernatant, both at pH 7.5. Hexokinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase (6), citrate synthase (7) malate dehydrogenase (8), and β-glucosidase (9) were further assayed. Protein in both the cell-free extract and the medium was estimated according to Lowry *et al.* (10) with a correction for the alkaloid content. The enzyme activities were expressed in mU, i.e., as nmoles of substrate converted in 1 minute per mg of protein in cell-free extract.

ANALYTICAL METHODS.—The content of total alkaloids was determined in the fermentation medium (11). Extracellular glucans were precipitated from culture filtrate with 4 volumes of acetone and the precipitate was dried to constant mass at 70°. The alkaloid spectra were estimated by means of high performance liquid chromatography (12). Lipids were extracted from the X-pressed mycelium twice by diethyl ether and then dried on the rotary evaporator.

## RESULTS

### 1. Submerged cultures of *C. purpurea* 129 with a dominant production of agroclavine.

The fermentation culture of the parent strain, further designated as 129 F, is a representative of these cultures. All its high-producing mutants were characterized by a similar development and alkaloid spectra.

#### 1.1. *Differentiation.*

The initial sphacelial filamentous mycelium changed to a sclerotia-like budding type during 7 days of fermentation; micro- and macroconidia were formed intensively (2). Terminal chlamydospores also occurred. In cultures older than 14 days, a suspension of spores and mycelial fragments predominated. Synthesis of clavine alkaloids proceeded in three phases: first production phase (P 1), transient phase (T), and second production phase (P 2) (fig. 1). In the P 1 phase, the extracellular glucans were extensively synthesized (fig. 2); their degradation occurred in the P 2 phase. Formation of cellular lipids proceeded mainly in phase T (fig. 1).

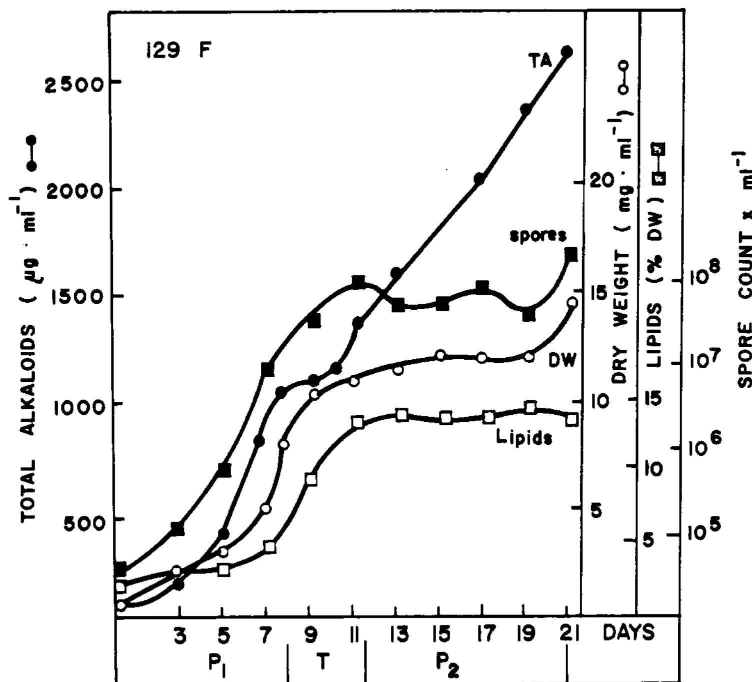


FIG. 1. Three-phase development of the culture 129 F. Note the delay in alkaloid synthesis in the phase T. (TA—total alkaloids, DW—dry weight).

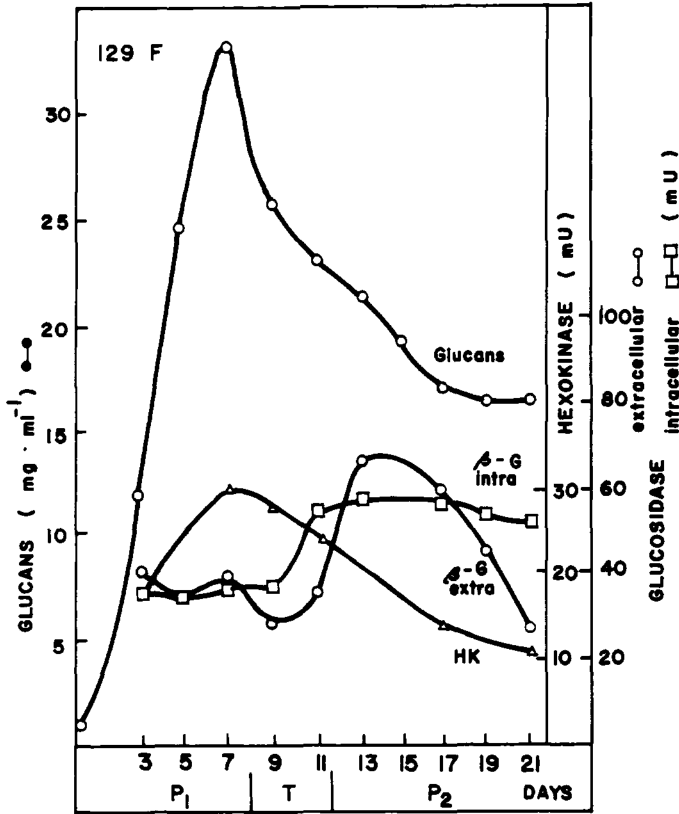


FIG. 2. Formation and degradation of glucans in the culture 129 F. (HK—hexokinase, β-glu—β-glucosidase).

1.2. *Enzyme apparatus.*

Fermentation phases P 1, T, and P 2—classified according to the kinetics of alkaloid production, biomass and glucan contents—were also characterized by differences in the enzyme apparatus of the culture. Hexokinase, the initial enzyme in the metabolism of saccharides, reached its maximum activity simultaneously with the maximum of glucan production, i.e., in a 7-day culture (fig. 2). In phase P 2, the activity of hexokinase slowly decreased, and the activity of extracellular and intracellular β-glucosidases simultaneously increased.

The course of the activity of glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, citrate synthase, and malate dehydrogenase was identical. It was characterized by a peak in phase P 1 as well as in phase P 2, and a decrease in phase T (fig. 3). The activity of glycolytic enzymes was very low.

Enzymes of the hexose monophosphate pathway, glucose-6- phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were detected only in phases T and P 2. In culture 129 F, the level of the latter enzyme was always higher than that of the former (fig. 4). Activity peaks of both enzymes corresponded well with the second maximum of glycolysis and that of malate dehydrogenase (fig. 7).

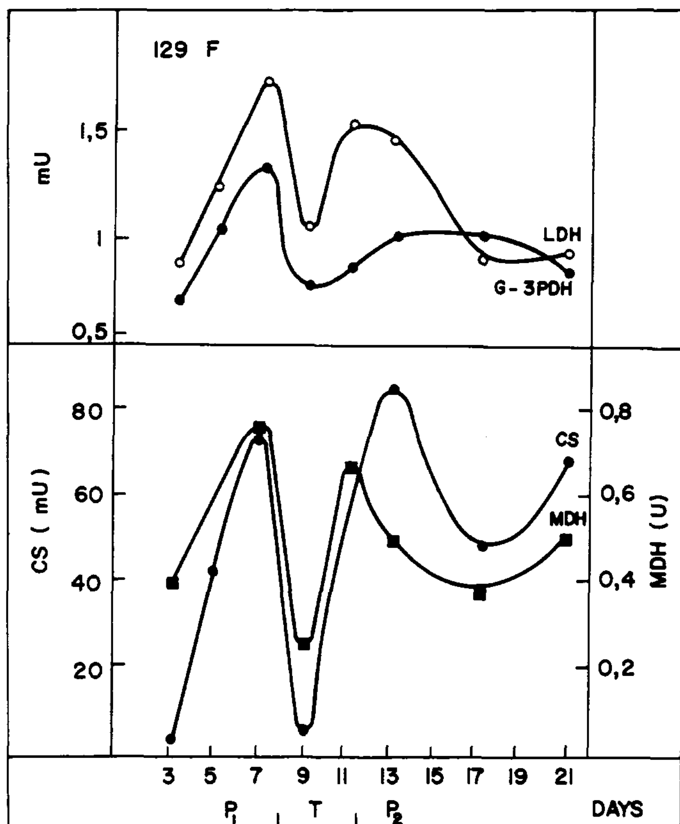


FIG. 3. Glycolytic and Krebs cycle enzymes during the development of the culture 129 F. Note a common trough of all enzymes in the phase T. (LDH—lactate dehydrogenase, G-3-PDH—glyceraldehyde-3-phosphate dehydrogenase, MDH—malate dehydrogenase, CS—citrate synthase).

## 2. Submerged cultures with a dominant production of elymoclavine.

Cultures of this type are represented by the vegetative inoculum of the strain 129 designated as 129 I and that fermentation culture of the mutant strain 244 designated as 244 F.

### 2a. *Differentiation and morphology.*

Classification of the culture development in three phases described in the fermentation of strain 129 holds, practically, also in cultures 129 I and 244 F (fig. 5). As compared with culture 129 F, the definite composition of the alkaloid mixture was set more slowly in cultures 129 I and 244 F, i.e., during 8–10 days (table 1). Morphology of culture 244 F was of a sclerotia-like type from the beginning of the cultivation, and the strain produced a number of terminal chlamydospores subjected to the microcycle (1). Cultures 129 I and F produced, as distinct from culture 244 F, exocellular glucans. It follows from this comparison that the morphological differentiation probably is not associated with the spectrum of alkaloids produced.

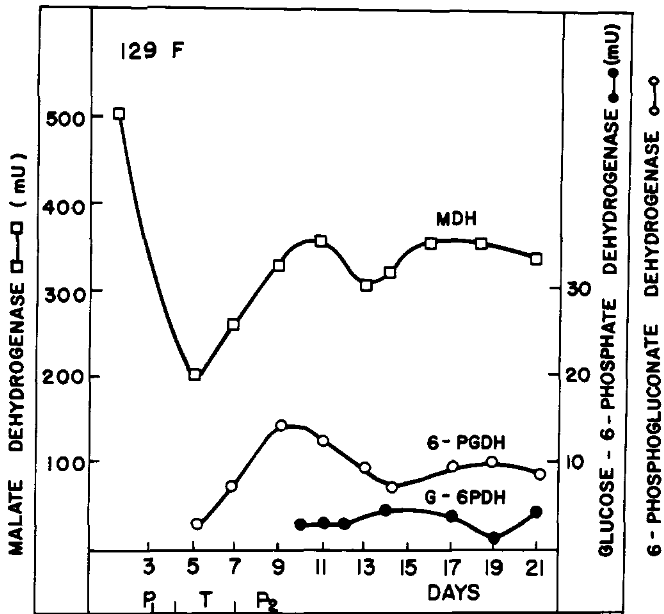


FIG. 4. Enzymes of the hexose monophosphate shunt in the culture 129 F. For the orientation in developmental phases the malate dehydrogenase activity is shown. These data are not taken from the experiment described in the figs. 1, 2, 3.

TABLE 1. Alkaloid spectra during submerged cultivations of *C. purpurea* strains.

| Day | culture 129 F | | | culture 129 I | | | culture 244 F | | |
|---|---|---|---|---|---|---|---|---|---|
| | agro | elymo | chano | agro | elymo | chano | agro | elymo | chano |
| | % | | | | | | | | |
| 2 | 77.8 | 22.2 | traces | | | | 80.1 | 19.1 | traces |
| 4 | 80.1 | 19.9 | — | 71.9 | 14.7 | 13.4 | 62.1 | 35.6 | 0.3 |
| 6 | 84.6 | 15.4 | — | 52.4 | 45.5 | 2.1 | 40.5 | 55.2 | 4.3 |
| 8 | 84.6 | 15.5 | — | 21.9 | 74.9 | 3.7 | 20.8 | 76.3 | 2.9 |
| 10 | 84.7 | 15.3 | — | 10.5 | 88.4 | 1.1 | 11.7 | 84.6 | 3.7 |
| 12 | 85.2 | 14.8 | — | 4.5 | 95.5 | — | 10.1 | 83.6 | 6.3 |
| 14 | 89.6 | 10.4 | — | 1.3 | 98.6 | — | | no | |
| 16 | 88.3 | 11.17 | — | | no | | | change | |
| 18 | 84.7 | 15.3 | — | | change | | | | |
| 20 | 87.2 | 12.8 | — | | | | | | |

Abbreviations: agro = agroclavine
elymo = elymoclavine
chano = chanoclavine

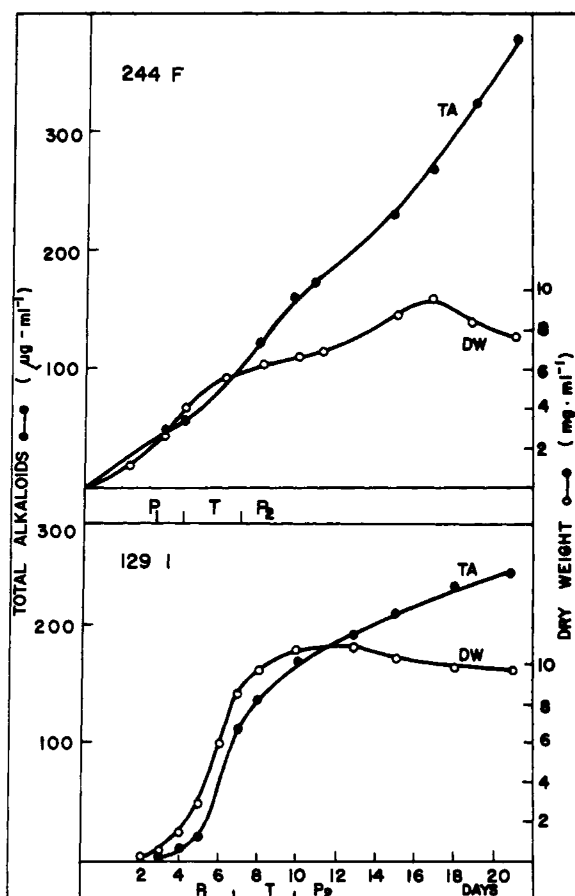


FIG. 5. Biomass growth and alkaloid synthesis in the elymoclavine type cultures. The developmental phases are not clearly distinguished with respect to alkaloid synthesis.

2b. *Enzyme apparatus.*

The enzyme apparatus of the cultures producing predominantly elymoclavine partially differed from that of the agroclavine-type cultures. This holds true particularly for the increased level of glyceraldehyde-3-phosphate dehydrogenase (by 1–2 orders of magnitude), whereas the level of lactate dehydrogenase remained at the level of culture 129 F (fig. 6). The increased activity of citrate synthase was also observed (fig. 7). In our cultures strain 244 exhibited the highest activities of glyceraldehyde-3-phosphate dehydrogenase, citrate synthase, and malate dehydrogenase. Similarly to culture 129 F, the glycolytic and Krebs cycle enzymes had a common course with simultaneous maxima. In culture 129 I, the maxima of malate dehydrogenase were slightly delayed with respect to those of citrate synthase.

The activity of hexokinase in culture 129 I corresponded roughly to that in culture 129 F, exceeding ten times the hexokinase activity in culture 244 F. The

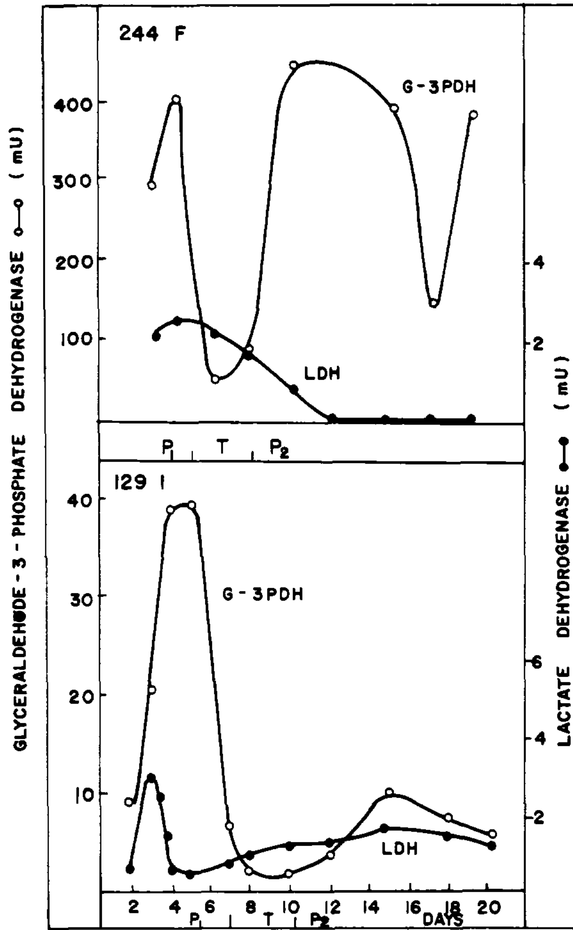


FIG. 6. Glycolytic enzymes of the elymoclavine type cultures.

exocellular β-glucosidase was produced in phases T and P 2 in culture 129 I; the content of glucans and enzyme activity were constant during phase P 2. Culture 244 did not contain glucans, the β-glucosidase activity was very low, and the level of hexokinase varied in accordance with enzymes of the oxidative pathways studied (fig. 8). The most pronounced difference between cultures of the agroclavine and elymoclavine type was observed in the onset and activity of the initial enzymes of the hexose monophosphate pathway (fig. 9). In the cultures producing elymoclavine, glucose-6-phosphate dehydrogenase could be detected from the beginning of the cultivation. In culture 129 I, the activity of 6-phosphogluconate dehydrogenase was relatively steady; the activity of glucose-6-phosphate dehydrogenase varied more, in agreement with the Krebs cycle enzymes. In culture 244 F, glucose-6-phosphate dehydrogenase increased almost in parallel with the alkaloid production to become the highest among the *C. purpurea* strains tested. The 6-phosphogluconate dehydrogenase activity of culture 129 I corresponded to that of culture 129 F. The activity of glucose-6-phosphate dehydrogenase in culture 129 I was five-fold.

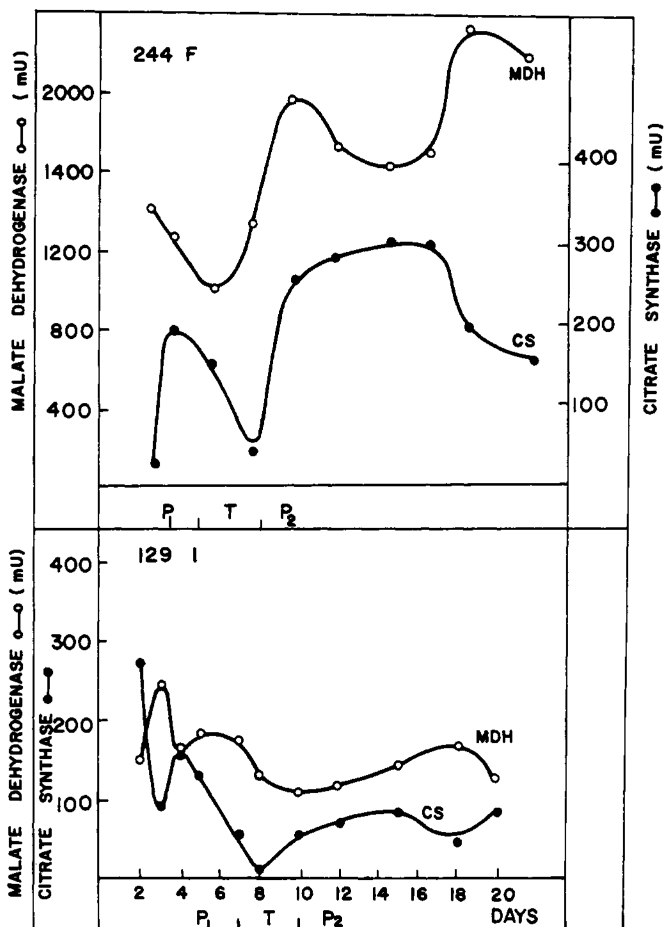


FIG. 7. Krebs cycle enzymes in the elymoclavine type cultures.

The effect of inorganic phosphate and citrate on the activity of glucose-6-phosphate dehydrogenase crude extract was also tentatively investigated. The enzyme was partially inhibited by both compounds (table 2). However, their effect was not additive. Both enzymes of the hexose monophosphate pathway were very sensitive to freezing and thawing. A single freezing and thawing of the crude extract resulted in a 99.8% loss of activity. The activity of glycolytic and Krebs cycle enzymes was not substantially changed by this treatment; the enzyme activities were all detected in freshly prepared cell-free extracts.

## DISCUSSION

The oxidative pathways of the saccharide metabolism represented by the Krebs cycle and hexose monophosphate pathway are of utmost importance for preservation of the metabolic balance in the axenic cultures of *C. purpurea*. This fact is demonstrated by the requirement for the exogenous citrate or another intermediate close to the Krebs cycle, which is necessary for maintaining the

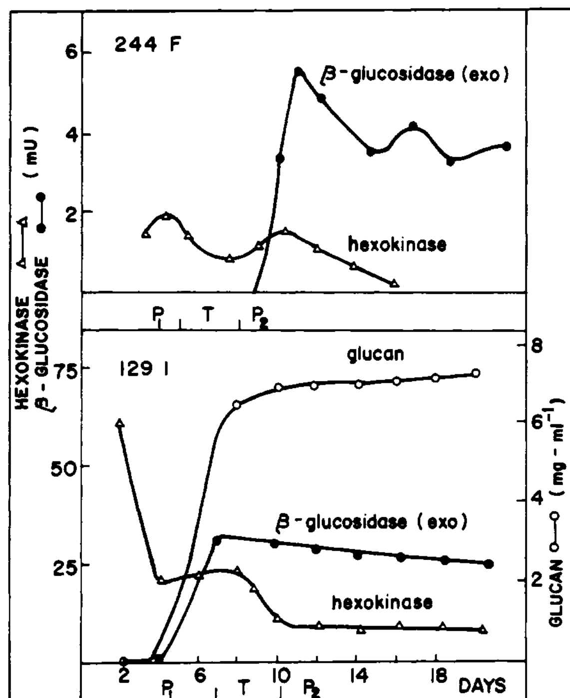


FIG. 8. Enzymes related to the glucan synthesis and degradation in the elymoclavine type cultures. Only exocellular β-glucosidase is shown.

culture growth and alkaloid production. The requirement for an organic acid related to the Krebs cycle is a common phenomenon in the genus *Claviceps* (13, 14), apparently due to the fact that parasitically growing ergot is sufficiently supplied with the Krebs cycle intermediates contained in the phloem sap of the host plant. Also the relationship between the activity of the hexose monophosphate pathway and the composition of the alkaloid mixture produced suggests a role for oxidative metabolism in alkaloid production.

Cultures of the agroclavine and elymoclavine type are characterized by variation of the oxidative pathway enzymes in accordance with the phases of culture development. The highest enzyme activities were detected in the first production phase P 1 and at the beginning of the second production phase P 2. The P 1 phase has, in general, higher requirements for respiration as a consequence of mycelial growth and conidiation (14). At present, it is not clear whether the decrease of the dehydrogenase levels in phase T is caused by an actual decrease of their activities or rather by dilution of the enzymes as a result of the synthesis of new enzymes required for the onset of the stationary phase of the culture development. The increase of the oxidative enzyme activities at the beginning of phase P 2 is probably associated with the completion of differentiation processes, including conidation. In this phase, degraded glucans serve as the energy source of culture 129 F and partially also of culture 129 I. The formation and degradation of glucans are positively correlated with the activities of hexokinase and β-glucosidase. We consider the low hexokinase activity in culture 244 F sufficient

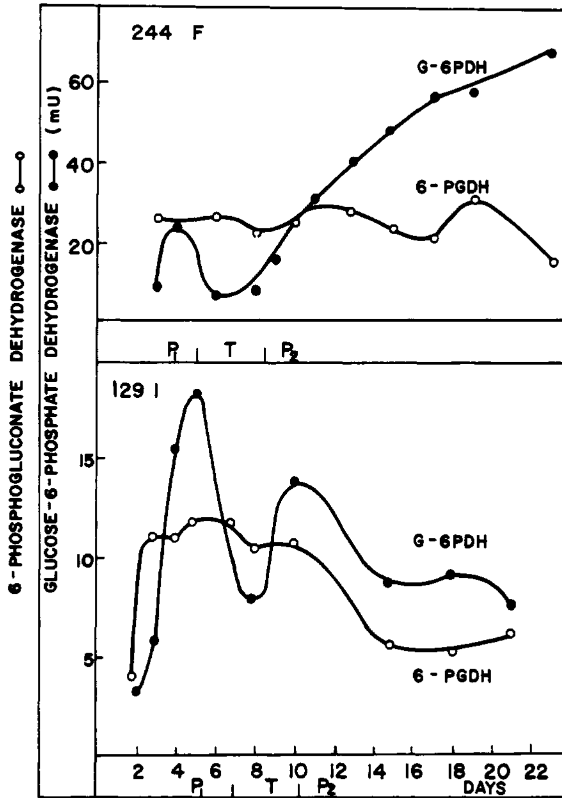


FIG. 9. Enzymes of the hexose monophosphate pathway in the elymoclavine type cultures.

TABLE 2. The effect of citrate and/or phosphate (mM) on the glucose-6-phosphate dehydrogenase activity (%).

| Citrate | Phosphate | Activity |
|---|---|---|
| — | — | 100 |
| 0.18 | — | 77 |
| 1.125 | — | 65 |
| 2.00 | — | 53 |
| — | 0.70 | 80 |
| — | 1.80 | 63 |
| 0.85 | 1.80 | 61 |
| 2.50 | 1.80 | 50 |

for feeding sugar catabolism pathways. Higher activities found in cultures 129 I and 129 F are necessary for polysaccharide synthesis.

The elymoclavine-producing cultures were characterized by the permanent presence and higher activity of the hexose monophosphate pathway generating the NADPH necessary for hydroxylation and oxygenase reactions. The increase of activity in the NADPH-forming pathway was accompanied also by higher activity of Krebs cycle enzymes. The sensitivity of glucose-6-phosphate dehydrogenase to citrate and phosphate inhibition might be of regulatory importance. However, without further studies this finding can serve only as a clue for avoiding experimental errors.

The activity of lactate dehydrogenase as compared with the activity of glyceraldehyde-3-phosphate dehydrogenase was very low. This pattern may indicate a harmony of elymoclavine synthesis with intensive oxidative metabolism, faster flow of glycolytic intermediates to the Krebs cycle, and higher NADPH formation. On the other side, no direct relationship between culture differentiation and morphology and the composition of the alkaloid mixture produced can be established in our strains.

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