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INDUCIBILITY OF PROGESTERONE HYDROXYLATING ENZYMES IN *RHIZOPUS NIGRICANS*

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SUMMARY

The induction of progesterone hydroxylases in *Rhizopus nigricans* was studied using chloramphenicol and cycloheximide as inhibitors of protein synthesis; effective inhibition of protein synthesis and enzyme induction was observed only with cycloheximide. Fungal mycelia not pretreated with the inducer do not hydroxylate progesterone if hydroxylation is assayed in the presence of cycloheximide indicating that the progesterone hydroxylating enzymes are inducible in this organism. Studies of the induction of progesterone hydroxylases in the growth medium and in the buffer solution revealed that the induction proceeds much faster in the buffer solution than in the growth medium.

INTRODUCTION

The hydroxylation of steroids by microorganisms has been known for a long time but is still relatively poorly understood although several microbial hydroxylations are industrially exploited for the production of steroid hormones. In our studies concerning the enzymes involved in the 11α-hydroxylation of progesterone in *R. nigricans* [1, 2] we were faced with the problem of whether the hydroxylases in this organism are inducible or not. In most fungi the enzymes involved in steroid hydroxylation are known to be inducible [3–7]. This has been, however, questioned for *R. nigricans* [7] since the experimental data from different research groups are not consistent. Sallam *et al.*[8] showed that preincubation of *R. nigricans* with progesterone gave better yields of hydroxylated products than non-preincubated cultures indicating that, in this organism, the hydroxylating enzymes are inducible. On the other hand, Nguyen-Dang and Janot[9], studying the influence of chloramphenicol on the induction of the progesterone 11α-hydroxylating system, did not find any effect of this drug on progesterone hydroxylation; they concluded, therefore, that progesterone hydroxylating enzymes in *R. nigricans* are not inducible. In the present report we show that protein synthesis in *R. nigricans* is prevented by cycloheximide and only slightly affected by chloramphenicol. The induction of hydroxylases in this organism was, therefore, studied using cycloheximide. The induction rate of the progesterone hydroxylating enzymes was followed in both the growth medium and in the sucrose-containing buffer.

* Systematic nomenclature for steroids in text: 4-pregnene-20-dione for progesterone and 11α-hydroxy 4-pregnene-3,20-dione for 11α-hydroxyprogesterone.

EXPERIMENTAL

Materials. Rhizopus nigricans ATCC 6227b was obtained from Chemical Institute Boris Kidrič, Ljubljana, Yugoslavia, through the courtesy of Mrs. E. Pertot. The chemicals were obtained from the following companies: progesterone*—Merck AG. Darmstadt, Germany; 11α-hydroxyprogesterone—Koch-Light, Colnbrook, England; cycloheximide—Calbiochem, San Diego, CA, U.S.A.; chloramphenicol—Pliva, Zagreb, Yugoslavia. All organic solvents (PA grade) were obtained either from Merck AG. Darmstadt, Germany, or from Riedel-de Haën AG. Seelze-Hannover, Germany, and were redistilled before use. All other chemicals were of the highest purity commercially available and were used without further purification.

Enzyme induction. Rhizopus nigricans was grown by aeration at 28°C for 46 h as described in our previous paper [1]. When the induction of hydroxylases was studied in the growth medium, progesterone was added to the cultures to a final concentration of 100 μg/ml 6 h before the end of mycelial growth. Enzyme induction in the buffer solution was studied as follows: after 40 h of mycelial growth the cells were harvested, washed thoroughly with cold distilled water, and resuspended in the same volume of 1 mM phosphate buffer pH = 5.5, containing 0.2 mM EDTA, 0.04 mM glutathione and 0.25 M sucrose [1]; the mixture was shaken for additional 6 h at 28°C with progesterone (100 μg/m final conc.) in order to induce the hydroxylases. When the effect of cycloheximide on the induction of hydroxylases was followed, the antibiotic was added together with the inducer at a final concentration of 200 μg/ml. The untransformed progesterone and its hydroxylated products were removed from the mycelia with cold saline as described by Shibahara *et al.*[10].

Assay procedure. Determination of hydroxylating activity was performed as described earlier [1]. The reaction products were extracted with chloroform and analyzed by thin layer and gas chromatography [1].

RESULTS

If the hydroxylases are inducible in *R. nigricans* one can expect that the progesterone-pretreated organism will contain a higher level of hydroxylating enzymes than the non pretreated one. The hydroxylating activity was, therefore, assayed in the noninduced mycelia and in mycelia which were pretreated with progesterone for several hours in the growth medium without using any antibiotic as a protein synthesis inhibitor. The results of this experiment are shown in Fig. 1. From the data presented one can see that the hydroxylating activity, if assayed at shorter intervals of time, is higher in the pretreated than in the not pretreated mycelia; after 6 h of incubation in buffer, the induced and the noninduced cultures transform essentially the same amount of the substrate. It is obvious that, in the absence of protein synthesis inhibitor during the assay procedure, the inducibility of hydroxylases can not be detected if the products of hydroxylation are measured 6 h or more after the addition of the substrate to the mycelial suspension. All subsequent enzyme assays were, therefore, performed under conditions in which protein synthesis was inhibited.

It is known that in some filamentous fungi protein synthesis is affected by antibacterial agents [11] whereas others are susceptible only to the antibiotics active in eucariotes [11]. In some fungi, however, both types of antibiotics can prevent the synthesis of inducible enzymes [11]. We selected one antibiotic

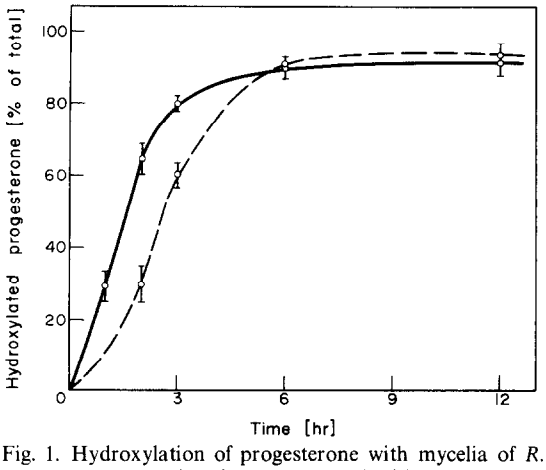

Fig. 1. Hydroxylation of progesterone with mycelia of *R. nigricans* pretreated and not pretreated with progesterone as inducer. Progesterone as inducer was added at 100 μg per ml of growth medium 6 h before the end of mycelial growth. 20 ml of cell suspension not pretreated (-----) and pretreated (——) with progesterone were assayed for hydroxylases by incubation with 3 mg of progesterone for different time intervals. The per cent conversion was obtained by gas chromatography analysis of chloroform extracts as described in the Methods.

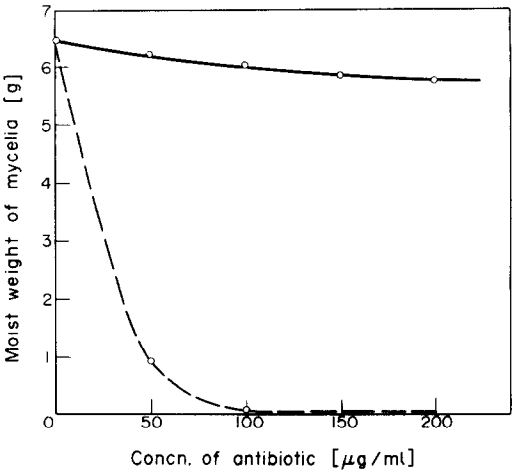

Fig. 2. Effect of chloramphenicol and cycloheximide on the mycelial growth of *R. nigricans*. The antibiotics were added together with the inoculum at the beginning of mycelial growth: chloramphenicol (——), cycloheximide (-----). After 46 h of incubation, the cells were washed with cold water, pressed between two filter papers, and wet weight per 100 ml was determined.

of either class, chloramphenicol and cycloheximide, in order to prevent mycelial growth of *R. nigricans*. Results of these experiments, presented in Fig. 2, show that cycloheximide inhibits the mycelial growth completely whereas chloramphenicol inhibits it only slightly. Chloramphenicol interferes most probably only with mitochondrial protein synthesis. We also studied the influence of chloramphenicol and cycloheximide on the inducibility of progesterone hydroxylases. For this purpose we measured the products of progesterone hydroxylation in mycelia incubated in the presence of progesterone without adding antibiotics and in the presence of either chloramphenicol or cycloheximide. The results of these experiments are presented in Table 1. From the data in Table 1 one can see that chloramphenicol had a negligible effect on the hydroxylation of progesterone whereas in the presence of cycloheximide the hydroxylation of progesterone was completely inhibited.

The results presented in Fig. 1 and Table 1 suggest that the induction of hydroxylating enzymes proceeds during the enzyme assay in the sucrose-containing buffer. In order to learn more about this phenomenon and to get a more direct proof for the inducibility of hydroxylases in *R. nigricans*, progesterone hydroxylation was followed in the presence of cycloheximide; fungal mycelia used as the source of hydroxylating enzymes were pretreated with progesterone either in the growth medium or in the sucrose-containing buffer. The results of this study are presented in Fig. 3. The noninduced mycelia, when tested in the presence of cycloheximide, do not hydroxylate progesterone at all. On the other hand, mycelia preincubated with progesterone for 6 h in the growth medium hydroxylate progesterone to some extent; the amount of hydroxylated products increases linearly with the time of incubation. When the induction of

Table 1. Hydroxylation of progesterone by *Rhizopus nigricans* in the presence of chloramphenicol or cycloheximide

Sample	% of progesterone hydroxylation 6 h incubation	12 h incubation
Control incubation	96.1 ± 0.5	99.6 ± 0.4
Incubation with chloramphenicol	85.5 ± 0.6	91.4 ± 0.4
Incubation with cycloheximide	<0.1	<0.1

The mycelia of *R. nigricans* were incubated with progesterone in the absence of antibiotics (control) and in the presence of chloramphenicol and cycloheximide (final conc. 200 μg/ml). The per cent conversion was determined according to the procedure described in Fig. 1.

hydroxylases is performed in the sucrose-containing buffer rather than in the growth medium, the amount of hydroxylases is about 10 times higher. The results presented in Fig. 3 show convincingly that the enzymes involved in progesterone hydroxylation in *R. nigricans* are inducible and that the induction proceeds much faster in the sucrose-containing buffer than in the growth medium.

Since the level of hydroxylating enzymes was so much higher if the induction was performed in the buffer rather than in the growth medium, the influence of the buffer *per se* on the constitutive level of the hydroxylases could not be completely excluded. A similar effect of incubation in buffer on the constitutive level of some enzymes was also observed in *Neurospora crassa* [12]. We, therefore, assayed the hydroxylases in *R. nigricans* after 6 h of incubation in the buffer without adding progesterone as inducer. The level of hydroxylating enzymes did not increase after such treatment.

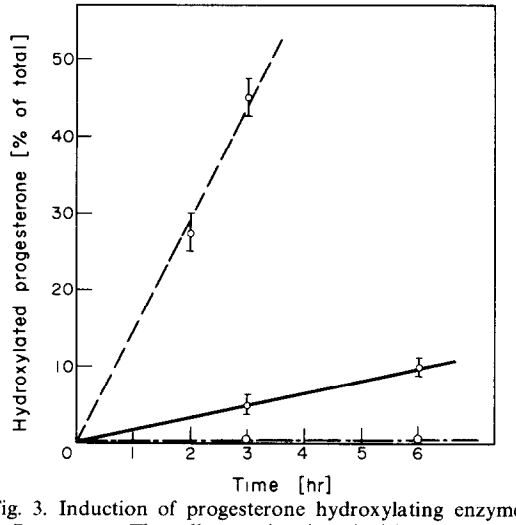

Fig. 3. Induction of progesterone hydroxylating enzymes in *R. nigricans*. The cells were incubated with progesterone as inducer at 100 μg/ml: (a) in the growth medium containing cycloheximide (200 μg/ml) (—·—·—), (b) in the growth medium without cycloheximide (——), (c) in the incubating buffer without cycloheximide (-----). Six h after induction the cells were washed with cold saline and hydroxylases were assayed in the presence of cycloheximide (200 μg ml) according to the standard procedure (see legend in Fig. 1).

DISCUSSION

The steroid transforming enzymes in fungi are generally inducible, their constitutive level being usually very low. Studies with cell-free preparations capable of hydroxylating steroids *in vitro* indicate a requirement for a certain induction time ranging from 2–20 h depending on the organism [3, 4, 11]. Our results show that noninduced, washed mycelium does not transform progesterone in the presence of cycloheximide whereas progesterone-induced mycelium under the same conditions hydroxylates the steroid. The level of hydroxylating enzymes constitutively present in the organism is less than we could detect by our method. This result convincingly proves the inducible nature of progesterone hydroxylating enzymes in *R. nigricans*. The difference between the conclusions of Nguyen-Dang and Janot[9] and ours in respect to the inducibility of *R. nigricans* could be explained by the fact that we might have studied another strain of the microorganism. The use of an improper inhibitor of protein synthesis by the above authors can not be excluded, however, since we could not show any effect of chloramphenicol on either enzyme induction or growth of the organism whereas cycloheximide inhibited both processes.

In our experiments the induction of progesterone hydroxylating enzymes was several times faster in the sucrose containing buffer than in the growth medium. A similar effect was observed also in *Rhizopus arrhizus* [6]. This phenomenon could explain why the induction of hydroxylating enzymes is difficult to detect in the mycelia of *R. nigricans* without the use of an appropriate inhibitor of protein synthesis. A relatively low level of progesterone hydroxylating enzymes induced in the growth medium suggests that the mycelia are not induced maximally under the conditions described. The difference between the rates of induction of steroid hydroxylating enzymes in the growth medium and in the buffer solution can not be explained on the basis of the results presented in the present paper. Experiments designed to elucidate this point are in progress since the observation described could help to explain the role of the induction of steroid hydroxylases in fungi.

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