DIRECT INHIBITION OF THE UPTAKE OF PROLINE BY CYCLOHEXIMIDE

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SUMMARY

Cycloheximide and puromycin completely inhibited the incorporation of L-proline into acid-insoluble protein by Achlya. P-fluorophenylalanine did not inhibit net incorporation but halted the synthesis of alkaline phosphatase activity. Of the three inhibitors of protein synthesis tested only cycloheximide immediately and completely interrupted the uptake of proline.

INTRODUCTION

Cycloheximide is a potent inhibitor of protein synthesis in eucaryotes (1). Furthermore, it has been demonstrated that this antibiotic is also an effective inhibitor of amino acid uptake (2,3,4,5). The inhibitory effect of cycloheximide on transport has been variously ascribed to a requirement for continued protein synthesis to replenish rapidly turning over permease systems (5), an increase in the concentration of cellular amino acids due to the inhibition of protein synthesis (3), and the labilization of membrane bound Ca^{2+} which is then complexed with PO_4^{2-} (2). The results presented here suggest that cycloheximide has a direct inhibitory effect on the uptake of L-proline by the water mold Achlya bisexualis.

EXPERIMENTAL PROCEDURE

Cultures. Culture medium - 1.25 g Bacto-peptone, 1.25 g Bacto-

TABLE 1

Inhibitor	ED_{50} (μM)	MD_{max} (μM)	I_{max} (%)
Cycloheximide	0.5	2.5	96
Puromycin	210	425	100
PFPA	20	150	94

Inhibition of dry weight yield. Inhibitors at varying concentrations were added to cultures at the time of inoculation and dry weights of mycelium determined after 20 hr of incubation. The 50% effective dose (ED_{50}), minimum dose giving maximum inhibition (MD_{max}), and the maximum inhibition (I_{max}) is given for each drug used in the study.

yeast extract, and 5.00 g D-glucose per liter of glass distilled water. Erlenmeyer flasks (250 ml) containing 100 ml of medium were inoculated with 2.5×10^4 spores of Achlya bisexualis (65-1) prepared as described elsewhere (6). These were incubated on a rotary shaker at 150 revolutions/min at 30°.

Inhibition of Growth. Cycloheximide, puromycin, and DL-p-fluorophenylalanine (PFPA) were added to duplicate cultures at the time of inoculation. After 20 hr these were harvested on pre-weighed Whatman 1 filter papers, dried at 60°, and re-weighed. Percent inhibition of dry weight yield was calculated by comparing treated and untreated cultures.

Uptake and Incorporation of Proline. To each 12 hr old culture 100 μCi of 5-^{3}H-L-proline (32.2 Ci/mmole) was added, and samples were withdrawn for measurement of uptake and incorporation. For uptake, 1 ml samples were collected on Whatman 3MM filter papers pre-soaked in 10 mM proline, washed with 10 ml of 0.5 mM $CaCl_2$ (0°), and dried at 60°. Incorporation into acid-insoluble protein was measured by withdrawing 10 ml samples, collecting as above, and placing the mycelium in 2 ml of 10% perchloric acid

(PCA) (0°). These samples were then washed by centrifugation twice with 2 ml of PCA, twice with 2 ml of 95% ethanol (0°), and were extracted for 15 min with 1 ml of 1 M NH_4OH at 80°. Aliquots of the extracts were then dried at 60° in scintillation vials. Radioactivity was determined by liquid scintillation counting as described elsewhere (7).

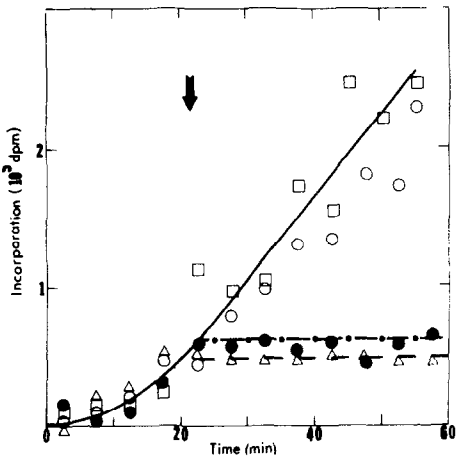
Measurement of Alkaline Phosphatase Activity. Ten ml samples from 22 hr old cultures were removed and the mycelium collected as above. Protein was extracted in a buffer containing 0.08 M Tris-Cl, pH 7.9 @ 4°, 6 mM $MgCl_2$, and 6 mM KCl (TMK) by grinding the mycelium in a Fisher tissue crusher. The brie was centrifuged for 15 min at 15,000 x g, and the supernatant assayed for alkaline phosphatase using a modification of the method of Garen and Levinthal (8). The reaction mixture contained 0.06 M Tris-Cl, pH 8.1 @ 25°, 5 mM KCl, 10 mM $MgCl_2$, and 5 mM p-nitrophenyl-phosphate. Assays were performed at 25°.

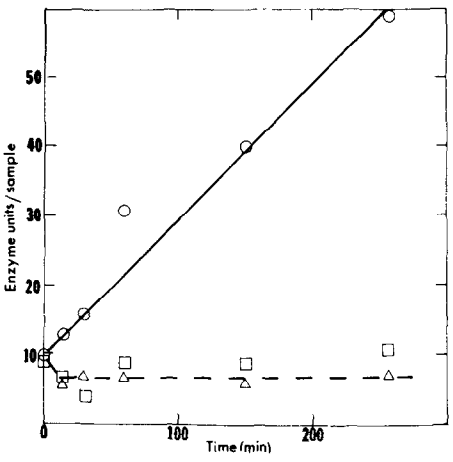
Reagents. Trizma base, puromycin-HCl, p-nitrophenylphosphate were obtained from Sigma; cycloheximide, PFPA from CalBiochem; and 5-^{3}H-L-proline from Schwarz/Mann. All other chemicals were reagent grade.

RESULTS

Inhibition of Growth. All three inhibitors decreased dry weight yield of Achlya. The 50% effective dose (ED_{50}), minimum dose giving maximum inhibition (MD_{max}), and the maximum inhibition of yield (I_{max}) are given in Table 1. In the experiments described below the MD_{max} was used for each drug.

Incorporation of Proline. Figure 1 shows that the incorporation of proline into acid-insoluble protein was linear after an initial lag of about 20 min. The addition of cycloheximide or





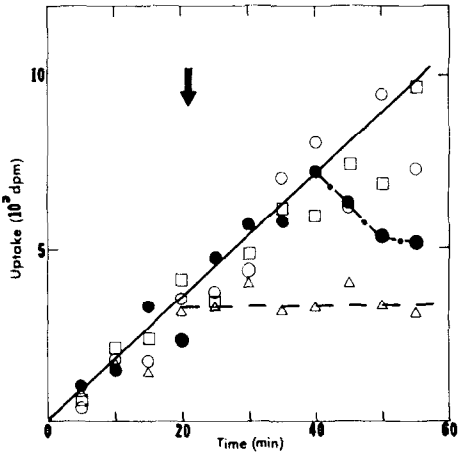


Figure 1. The incorporation of ^{3}H-proline into acid-insoluble protein. Isotope was added at time zero and samples were removed and prepared as described under Experimental Procedure. The arrow indicates the time of addition of the drugs. Incorporation is given per ml of culture. ○ , no addition; □ , PFPA; △ , cycloheximide; ● , puromycin.

Figure 2. The effect of PFPA and cycloheximide on the synthesis of alkaline phosphatase. Inhibitors or water were added to exponentially growing cultures at time zero. Samples were removed at times after the addition and protein prepared as described under Experimental Procedure. Alkaline phosphatase activity was assayed by the production of nitrophenol from p-nitrophenylphosphate by measuring increase in absorbancy at 405 nm. One enzyme unit is 1 nmole of nitrophenol produced per min at 25°. ○ , distilled water; △ , cycloheximide; □ , PFPA.

Figure 3. The uptake of ^{3}H-proline. Isotope was added at time zero and samples were removed and prepared as described under Experimental Procedure. The arrow indicates the time of addition of the drugs. Uptake is given per ml of culture. ○ , no addition; □ , PFPA; △ , cycloheximide; ● puromycin.

puromycin immediately stopped further incorporation. PFPA, on the other hand, had little effect on net incorporation.

Effect of PFPA. In order to ascertain the effect of PFPA on the synthesis of viable protein, alkaline phosphatase activity was measured after the addition of the inhibitor. Cycloheximide and distilled water were added to control cultures at the same time as PFPA. The results of this experiment are shown in Figure 2. Both cycloheximide and PFPA completely inhibited the synthesis of active alkaline phosphatase, demonstrating that, although PFPA did not inhibit net incorporation of proline into protein, it did in fact inhibit the synthesis of functional enzymes.

Uptake of Proline. Figure 3 shows the effect of the three drugs used in this study on the uptake of proline. With no addition, uptake was linear for at least 55 min. Uptake of ^{3}H-proline was 3-5 times greater than incorporation. PFPA had no effect, while puromycin inhibited uptake only after 20 min. Cycloheximide inhibited uptake immediately and completely.

DISCUSSION

Cycloheximide and puromycin are effective inhibitors of protein synthesis in Achlya. Although PFPA does not inhibit the net incorporation of proline into protein, it does stop the synthesis of functional enzymes. The fact that, of these three inhibitors of protein synthesis, only cycloheximide completely and immediately inhibits the uptake of proline shows that its effect on uptake cannot be explained by its inhibition of protein synthesis. Furthermore, it is unlikely that the inhibition of uptake was due to the efflux of Ca^{2+} from the cell membrane. Cameron and LéJohn (9) observed that in Achlya methionine uptake was completely inhibited by citrate (a chelator of divalent cations) but unaffected by cycloheximide. They also observed

that $^{45}Ca^{2+}$ uptake in a medium containing glucose and yeast extract was not inhibited for 120 min after the addition of cycloheximide. We conclude that cycloheximide very likely directly inhibits the uptake of L-proline by Achlya.

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