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Action of Patulin on a Yeast

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The action of patulin on *Saccharomyces cerevisiae* was studied. At weak doses, the drug inhibited growth, but inhibition was transient. After 10 min, syntheses of rRNA, tRNA, and probably mRNA were blocked; this was shown by radioactive precursor incorporation assays and gel electrophoresis of RNAs. After recovery of growth, patulin disappeared from the medium. It seemed that this degradation resulted from the activity of an inducible enzymatic system. Induced cells resisted very high patulin concentrations.

Patulin is a mycotoxin produced by numerous molds, especially *Penicillium patulum* and *Byssochlamys nivea*. This mycotoxin has been identified in various fruit juices and principally in apple juice and silage (1, 4, 11, 13). The biosynthesis of patulin by *Penicillium urticae* has been studied (6), and a great deal of data have shown that it may be produced in very abundant yields (up to 50% of the biomass increase [14]); however, no function has been discerned for this toxin. The action spectrum of patulin is very broad, including most bacteria and animal and vegetable cells. Neither the primary target nor the mechanism of action of the toxin has been specified (9, 10, 12). Many enzymes having the sulfhydryl group in their active sites are sensitive to patulin, but enzymes without the sulfhydryl group can be equally inhibited (12). Mouley and Hatey (10) reported the inhibition of RNA polymerase A activity in isolated rat liver nuclei. Our purpose in this study was to specify the action of patulin on *Saccharomyces cerevisiae*. We chose this yeast because of the well-documented fact that patulin disappears during fermentation of apple juice; so far, no model to explain this degradation has been proposed.

MATERIALS AND METHODS

Strain. The *S. cerevisiae* strain used in this work (1278b [α]) is a haploid strain and has been described by Grenson et al. (5).

Medium. We used minimal medium 149, which contains 3% glucose and has been previously described (5).

Incorporation test. Protein synthesis was measured by determining the incorporation of [^{3}H]leucine and [^{3}H]lysine. Yeast cells were grown at 29°C in minimal medium with (per milliliter) 0.4 μCi of [^{3}H]leucine, 0.4 μCi of [^{3}H]lysine, 100 μg of cold L-leucine, and 100 μg of L-lysine. At intervals, 1 ml of culture was boiled for 30 min in 5 ml of 10% trichloroacetic acid; precipitates were collected on HA membrane filters (0.45-μm pore size; Millipore Corp.), and radioactivity was measured.

For determining RNA synthesis, the culture was grown at 29°C in minimal medium 149; at log phase, [^{3}H]uracil (0.4 μCi/ml; specific activity, 53 Ci/mmol) or [^{14}C]adenine (0.4 μCi/ml; specific activity, 53 Ci/mmol) was added. At intervals, the amount of radioactivity incorporated was determined as previously described (16).

Labeling of cells for RNA analysis, RNA extraction, and discontinuous polyacrylamide gel electrophoresis (2.4 to 7%) were performed as described previously (16).

Patulin. Patulin was produced by *P. patulum* NRRL 2159A and purified as previously described (14). It was identified by high-performance liquid chromatography and infrared spectrum and melting point studies; commercial patulin (P 1639; Sigma Chemical Corp.) was used as the reference. Patulin was assayed by high-performance liquid chromatography. A Hewlett-Packard model 1084 B chromatograph with a variable-wavelength UV detector, operating at 277 nm, was used. All solvents were of spectrophotometric grade. Analysis was carried out with a 10-μm Lichrosorb (Hibar) silica column (E. Merck AG). The mobile phase was chloroform-cyclohexane-ethanol-acetonitrile (25:7.5:1.5:1 [vol/vol/vol/vol]), the sample volume was 10 μl, the solvent flow rate was 2 ml/min, and the patulin retention time was 2.91 min. The culture samples were centrifuged, filtered (0.45-μm HA filters [Millipore]), and then injected without previous extraction or purification (17).

RESULTS

Action of patulin on RNA synthesis. In the first set of experiments, patulin (0 to 100 μg/ml) and [^{3}H]uracil (0.4 μCi/ml) were added simultaneously to a log-phase culture. At various times, samples were withdrawn as described above. A decrease of at least 50% in the uracil incorporation rate was observed after 10 min of incuba-

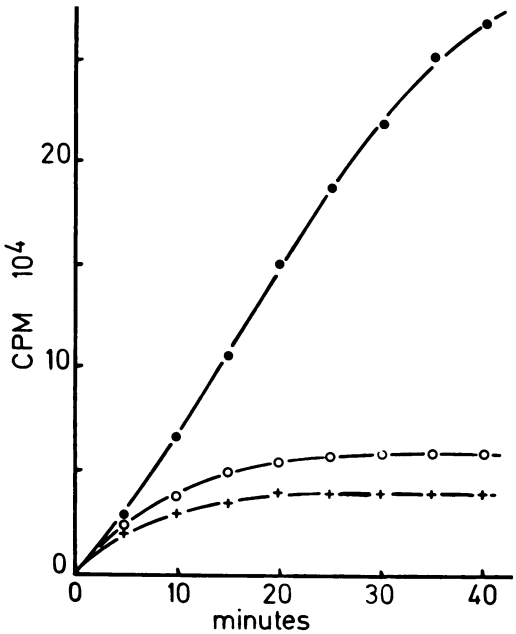

FIG. 1. Uracil incorporation into RNA in the presence of patulin at 0 μg/ml (●), 50 μg/ml (○), or 100 μg/ml (+).

tion in the presence of the drug (50 and 100 μg/ml) (Fig. 1).

In the second set of experiments, patulin (50 μg/ml) was added at zero time, and the radioactive precursor ([^{14}C]adenine; 0.25 μCi/ml) was added after 0, 4, 9, and 17 min. Total radioactivity in the cells (adenine pool plus RNA incorporation) and RNA incorporation were measured as functions of time. (DNA incorporation was not taken into account since DNA represented <2% of the total nucleic acids.)

The transcription machinery was rapidly inhibited (Fig. 2). The size of the adenine intracellular pool indicated that nucleotide uptake was less affected by patulin than was the RNA incorporation.

Analysis of RNA synthesized in the presence of patulin. An *S. cerevisiae* culture grown at 29°C was divided into several portions. Various amounts of patulin (0, 25, 50 μg/ml) and a constant amount [^{3}H]uracil (10 μCi/25 ml) were added. Cells were harvested after 3 h, and RNAs were extracted and analyzed by polyacrylamide gel electrophoresis (16) (Fig. 3). It is clear that the syntheses of rRNA and tRNA species were totally inhibited in the presence of 50 μg of patulin per ml. At 25 μg of patulin per ml, inhibition was not complete, but there was no preferential inhibition of either RNA species.

Protein synthesis was slightly affected at 25 μg/ml. At 50 μg/ml, the reduction of the amino acid incorporation rate was consistent with an mRNA half-life of 30 min (15).

Degradation of patulin by *S. cerevisiae* in vivo. We added 50 μg of patulin per ml to a log-phase culture; after a transient inhibition period, growth and RNA synthesis resumed at a rate slightly slower than that seen in the absence of patulin (Fig. 4). During growth, there was an important decrease in the patulin concentration in the medium (Fig. 5). This decrease, although not strictly parallel with growth, was associated with it; cells killed by heat (10 min at 80°C) did not induce the reduction of the patulin concentration (Fig. 6). Inhibition of protein synthesis,

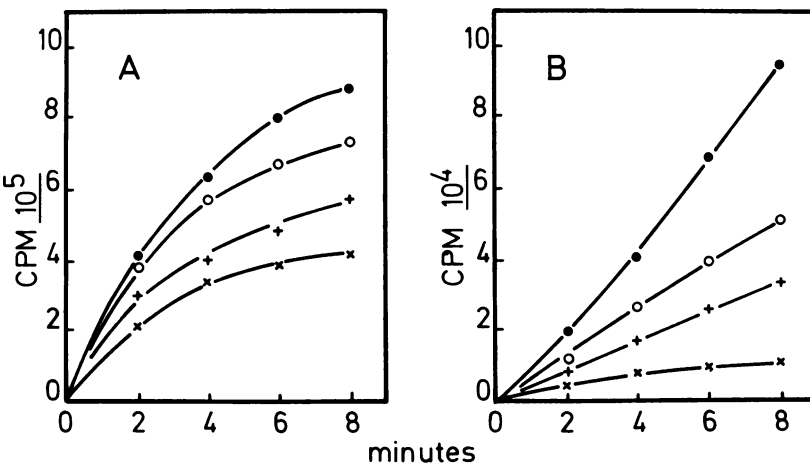

FIG. 2. Influence of patulin on [^{14}C]adenine incorporation as a function of time. (A) Total radioactivity of cells (adenine pool plus RNA incorporation); (B) RNA incorporation (trichloroacetic acid-precipitable materials). Patulin was added at zero time, and adenine was added at 0 min (●), 4 min (○), 9 min (+), or 17 (×) min after the addition of patulin.

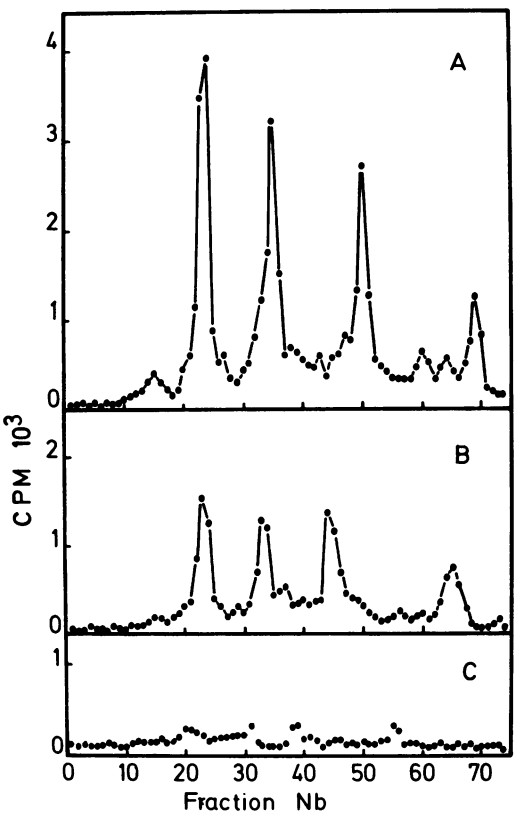

FIG. 3. Influence of patulin at 0 μg/ml (A), 25 μg/ml (B), or 50 μg/ml (C) on rRNA and tRNA syntheses. The technique of discontinuous polyacrylamide gel electrophoresis (2.4 to 7%) and the correction procedure for RNA losses during extraction have been described previously (16).

effected by adding 2 μg of cycloheximide per ml at the same time that patulin was added, prevented the disappearance of the mycotoxin. Nevertheless, if cycloheximide was added 3 h after the addition of patulin, the degradation rate was slowed down but not stopped (Fig. 6).

Effect of patulin concentration. Log-phase cells and cells preincubated for 3 h in the presence of 50 μg of patulin per ml were incubated with 12.5 to 500 μg of patulin per ml. Patulin degradation was measured after 6, 12, 18, 24, and 48 h (Fig. 7). At 12.5, 25, and 50 μg/ml, the patulin degradation rate was slightly higher in preincubated cells; at 200 and 500 μg/ml, inactivation of patulin was noteworthy in preincubated cells, but no or nearly no degradation occurred in the reference culture. This was a consequence of the fact that preincubated cells were resistant and grew in the presence of higher doses of patulin, whereas non-preincubated cells did not grow.

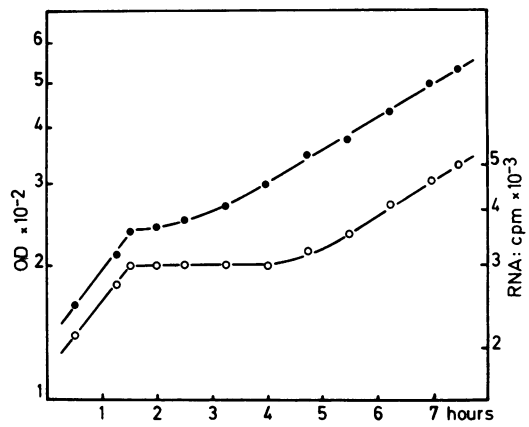

FIG. 4. Influence of patulin (50 μg/ml) on yeast growth (●) and RNA incorporation (○). Patulin was added at 90 min. Yeast growth was measured by optical density determinations (540 nm), and incorporation in RNA was measured as described in the text.

DISCUSSION

Although patulin was discovered in the 1940s, its inhibition mechanism is not yet known. Some facts have been demonstrated, but the primary target of the toxin remains undiscovered. Patulin reacts with sulfhydryl groups (2, 3, 8); thus, many enzymes having a sulfhydryl group at their active sites are inhibited by patulin, but this is not a general rule, and there are sensitive enzymes which do not possess such a group.

Another hypothesis has been proposed by Mouley and Hatey (10); these authors have shown the inhibition of RNA polymerase A in isolated rat liver nuclei. RNA polymerase B (responsible for mRNA synthesis) is less sensitive; nevertheless, the dose of patulin used was very high (200 μg/ml for 50% inhibition of RNA polymerase A). Using this system, Hatez and Gaye have also shown inhibition of protein synthesis (7). Inhibition of *S. cerevisiae* growth by patulin is transient, and the duration is proportional to the dose of the toxin (15).

There are thus two stages in the action of patulin on yeast cells: first, the action of the toxin on yeast metabolism and the subsequent inhibition of growth; second, the resumption of growth, indicating the appearance of a resistance mechanism probably associated with the previously reported disappearance of the mycotoxin during apple juice fermentation.

Uracil incorporation into trichloroacetic acid-insoluble material (ca. 10 min) was rapidly stopped at patulin concentrations of >12.5 μg/ml. This rapid cessation obviously indicated an arrest of the synthesis of rRNA, the major component of the total RNA (>80%). To draw a conclusion about tRNA and mRNA on the basis

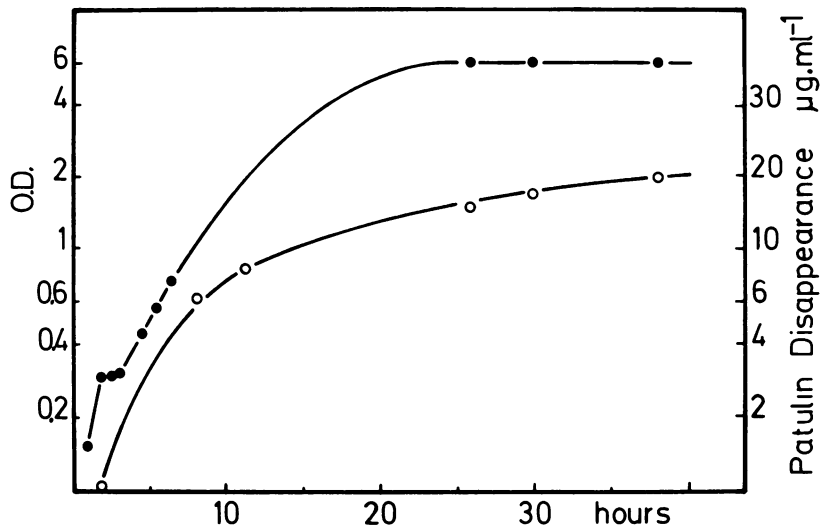

FIG. 5. Patulin degradation (○) during growth (●). Patulin (50 µg/ml) was added to a log-phase culture at 90 min and measured as described in the text. Growth was measured by optical density determinations (540 nm).

of the results of this incorporation test would be hazardous. The problem has been analyzed by polyacrylamide gel electrophoresis and, indirectly, by studying the kinetics of radioactive amino acid incorporation. The syntheses of rRNAs (25S, 17S, and 5S) and tRNAs (4S) were equally inhibited (Fig. 3). The kinetics of radioactive amino acid incorporation (into tricloroacetic acid-precipitable materials) after the addition of 50 µg of patulin per ml were consistent with an mRNA half-life of 30 min (data not shown). This behavior is similar to the pattern observed for thermosensitive *S. cerevisiae* mutant strains 4572, 4472, and 1564 (16) and qualitatively similar to the behavior induced by daunomycine and lomofungin, which are known to inhibit RNA synthesis. However, the modes of action of lomofungin and patulin are not similar: lomofungin is a chelating agent with an action which can be reversed by Mg^{2+}. Patulin's action

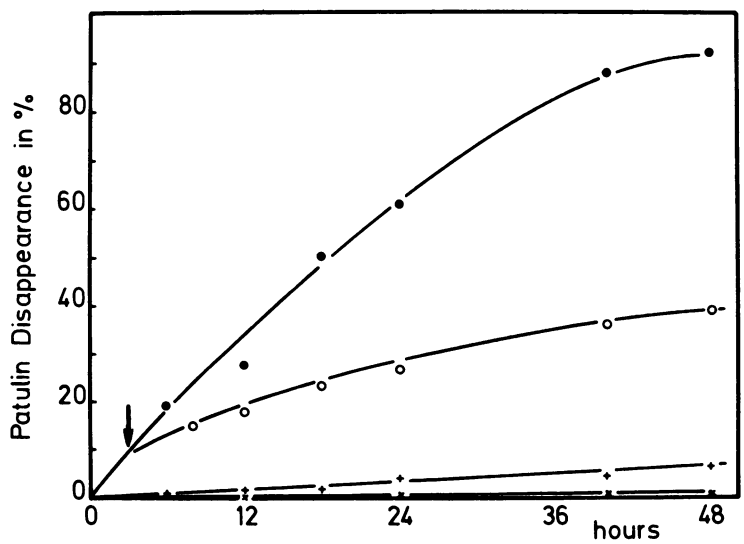

FIG. 6. Patulin degradation. Symbols: ●, 50 µg of patulin per ml added; ○, 50 µg of patulin per ml added; 2 µg of cycloheximide per ml added 3 h after addition of patulin (arrow); +, 50 µg of patulin per ml and 2 µg of cycloheximide per ml added at the same time; ×, 50 µg of patulin per ml added; cells killed by heat (10 min at 80°C).

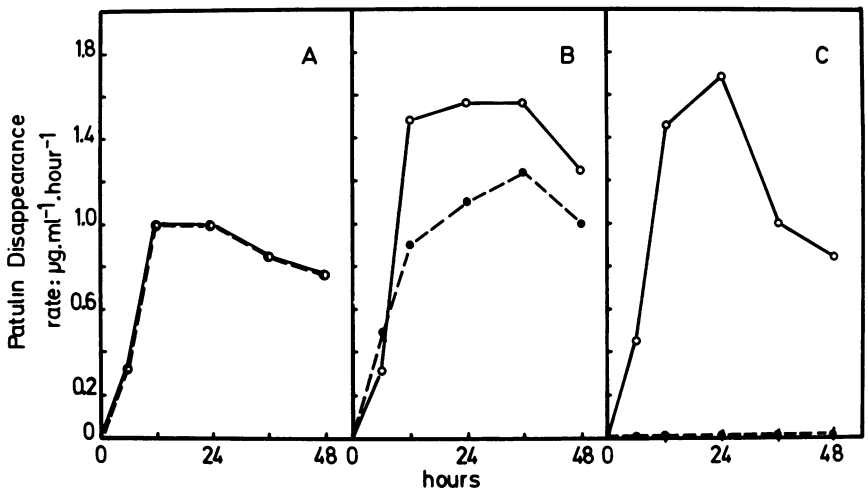

FIG. 7. Patulin disappearance. Symbols: ○, cells preincubated for 3 h with 50 μg of patulin per ml; ●, non-preincubated cells with patulin at 50 μg/ml (A), 100 μg/ml (B), or 500 μg/ml (C).

is not reversed by 20 μg of Mg or Mn per ml (data not shown). These arguments strongly favor the hypothesis that the primary target of patulin is the transcription mechanism.

Disappearance of patulin during fermentation is a well-known phenomenon: neither a mechanism for it nor a lag period preceding growth resumption has ever been reported. Our results show that patulin degradation is an active process and a consequence of yeast metabolism: pasteurized yeasts were incapable of provoking the disappearance of the toxin (Fig. 6). The inducible character of the phenomenon was further proven by the results obtained for patulin in the presence of cycloheximide, an antifungal agent which blocks peptide bond formation. When patulin and cycloheximide were added simultaneously to a culture, protein synthesis was blocked and no toxin was degraded (Fig. 6). These results indicate that the wild-type strain was incapable of spontaneous patulin inactivation and that detoxication requires protein synthesis.

When cycloheximide was added 3 h after the addition of patulin, degradation continued but at a slower rate (Fig. 6), possibly indicating that the newly synthetized proteins have a catalytic rather than stoichiometric role.

At patulin concentrations of >200 μg/ml, growth was definitely blocked and resistance induction was impossible. This could be a consequence of complete inhibition of protein synthesis by patulin at high concentrations. However, if the cells are preinduced for 3 h at 50 μg/ml, they become resistant to doses of >200 μg/ml. Patulin-sensitive mutants in which patulin degradation is no longer inducible have been isolated (P. Thonart and J. Bechet, Vème Int. Symp. Yeast 1980, p. 225–230). The present experiments did not enable us to determine if the induced protein(s) acts directly on patulin or permits the synthesis of a detoxifying substance(s). This question is being studied.

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