In the presence of NADPH and CuCl₂, patulin induced the cleavage of ColEl DNA and λ phage DNA in vitro. The DNA-cleaving activity of patulin was concentration dependent. At the lowest concentration of patulin, ColEl supercoiled DNA was relaxed and the highest concentration induced linearization of the DNA. This activity was inhibited by superoxide dismutase, catalase and radical scavengers, showing involvement of free radicals in the DNA-cleavage. λ Phage DNA was also degraded by patulin under the same conditions.

Patulin is a secondary metabolite of toxigenic strains of Penicillium, Aspergillus and Byssochlamys species. After its isolation in the early 1940s it was regarded as an antibiotic but as its toxicity for animals became recognized it was classified as mycotoxin.

Patulin is stable at a pH below 7 and readily degrades at alkaline pH. This is probably one of the reasons why it is found frequently as a contaminant of fruits or fruit products like apple juice or cider. As it is able to form adducts with thiol-group containing compounds, it may react with many cellular constituents, especially thiol-group containing proteins. However, until now its mode of action has not been clear. Its importance as a food contaminant is also not very well established. There are unconclusive reports of mutagenic and carcinogenic properties. According to Umeda et al., patulin causes single- and double-strand breaks in the DNA of FM3A and HeLa cells. However, Frank and Becci et al. could not find an increase in tumors after long-term feeding experiments with rats. In short-term mutagenicity tests, patulin was negative in the Ames test but positive in the rec-test, we searched for an explanation for its DNA-attacking ability. An attempt to bind ¹⁴C-labeled patulin (1 mCi/mm) to DNA failed. Thus, covalent binding to or intercalation into DNA seemed unlikely.

Strand scissions of DNA without covalent binding have been described for compounds such as adriamycin, daunorubicin, dactinomycin and bleomycin. Therefore, we investigated the action of patulin under conditions similar to those used with these antibiotics.

In the presence of Cu⁺⁺-ions and reducing agents like NaBH₄ or NADPH, patulin was able to cleave ColEI plasmid DNA and λ phage DNA. This effect could be prevented or diminished by superoxide dismutase and catalase, and by radical scavengers, indicating the involvement of free radicals in the DNA-attacking activity of patulin.

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2 Own, unpublished observations.
Materials and Methods

Patulin was prepared by extracting the culture medium of *Penicillium patulum* NRRL 5259 with EtOAc and was purified on acid (pH 4) alumina columns. As a medium for the production of patulin, commercial apple juice was used. This yielded 3 mg/ml of patulin by inoculation with $1.2 \times 10^6$ spores/ml and shaking (100 rpm) at 28°C for 8~10 days. Patulin was crystallized from ethyl ether and its purity was checked by thin-layer chromatography. It was visualized with the 3-methylbenzothiazolinone-2-hydrazone (MBTH)-reagent. The purity was estimated by calculating the ratios of absorbances at 260 nm and at 276 nm. This ratio is 0.7 with pure patulin. ColEl plasmid DNA was prepared from *E. coli* JC 411 (Thy−) by the conventional ethidium bromide procedure, and λ phage DNA was obtained from *E. coli* CSH 44 lysogenic for a λ phage carrying the cI857 mutation. Purification was carried out according to a literature procedure. All chemicals were from Merck (Darmstadt), Sigma (Munich), or Serva (Heidelberg) and they were of the purest grade available. Superoxide dismutase and catalase were from Boehringer (Mannheim).

Agarose gel electrophoresis of DNA was performed with 0.7% gels in a submarine chamber. The total volume of strand scission reaction mixtures was 30 µl. Their detailed compositions are given in the legends to figures. After the reaction mixtures had been incubated at 37°C for 2 hours, they were mixed with 3.3 µl of 0.025% bromophenol blue in 40% sucrose and brought into the wells of the gel. Tris-acetate (TAE) buffer was used for electrophoresis which was carried out at 120 volts and 60 mA for 4 hours with cooling of the gel support. For staining DNA, the gel was submerged into 0.2% ethidium bromide in TAE buffer for 1 hour and destained by placing it in water for 3 hours. The DNA bands were detected by fluorescence using long wave UV light. Supercoiled ColEl plasmid DNA could be distinguished from the linear or nicked circular (relaxed) form by different electrophoretic mobility.

Results and Discussion

Fig. 1 shows a gel in which ColEl plasmid DNA was separated after reaction with patulin, CuCl$_2$ and NADPH. None of these compounds alone gave a visible reaction with the DNA at the concentrations indicated. However, a combination of 0.5 mM patulin with 1.5 mM CuCl$_2$ caused some re-
Fig. 2. Effect of radical scavengers on the DNA-cleaving activity of patulin. Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 28 µg ColE1 DNA/ml, 1 mM NADPH, 1.5 mM CuCl₂ and 0.25 mM patulin. Lane 13: control; Lane 1~3: superoxide dismutase (SOD) at 33.5 µg/ml (3), 16.8 µg/ml (2), 6.7 µg/ml (1); lane 4~6: mannitol (MAN) at 415 mM (6), 207 mM (5), 83 mM (4); lane 7~9: catalase (CAT) at 33.5 µg (7), 16.8 µg (8), 6.7 µg (9); lane 10~12: dimethyl sulfoxide (DMSO) at 2.34 mM (10), 1.17 mM (11), 467 mM (12).

Fig. 3. Effect of patulin on λ phage DNA in the presence of CuCl₂ and NADPH. Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 4.16 µg λ DNA/ml, CuCl₂, NADPH and patulin. Lane 1: control, containing only λ DNA; lane 2: 1 mM patulin; lane 3: 1 mM NADPH; lane 4: 1 mM NADPH, 1 mM patulin; lane 5: 1.5 mM CuCl₂; lane 6: 1.5 mM CuCl₂, 1 mM patulin; lane 7: 1 mM NADPH, 1.5 mM CuCl₂; lane 8: same as lane 10; lane 9~13: 1 mM NADPH, 1.5 mM CuCl₂, 0.5 mM (9), 1 mM (10), 5 mM (11), 10 mM (12), 14 mM (13) of patulin.

Relaxed
cccDNA

laxation of the supercoiled DNA as, to a lesser extent, did a combination of NADPH and CuCl₂. Combinations with constant CuCl₂ and NADPH concentrations and varying patulin concentrations caused cleavage of the DNA. The extent of cleavage depended on the patulin concentration. Though NADPH is physiologically more significant NaBH₄ could replace NADPH as a reducing agent in the DNA cleavage reaction with patulin and CuCl₂.

In the concentration range between 0.25 mM and 0.5 mM patulin, most of the cccDNA (covalently closed circular DNA) was relaxed, indicating single-strand breaks, whereas at concentrations from 1 mM upwards patulin linearized the DNA, i.e. caused double-strand breaks. The lowest dose which
brought about single-strand breaks is in the range which also brings about single-strand breakages of chromosomal DNA in living E. coli cells\(^{10}\). The action of doxorubicin and other anthracyclic and benzoquinoid antibiotics on DNA can be prevented with superoxide dismutase, catalase and radical scavengers\(^{12,13}\). This is also the case with patulin (Fig. 2). Superoxide dismutase strongly diminished relaxation of supercoiled DNA at concentrations of 33.5 \(\mu g/ml\). Catalase at a concentration of 33.5 \(\mu g/ml\) was also very effective. Mannitol reduced most efficiently cleavage at all concentrations but did not prevent it completely, whereas dimethyl sulfoxide (DMSO) had only a slight effect on the DNA-cleaving activity of patulin.

\(\lambda\) Phage DNA was also attacked by patulin (Fig. 3). With this linear DNA, no effect was found with patulin, \(\text{CuCl}_2\) or NADPH alone or with combinations of always two of these compounds. However, in the presence of \(\text{CuCl}_2\) and NADPH the DNA was increasingly damaged as the patulin concentration was raised from 1 \(\text{mm}\) to 14 \(\text{mm}\). The tailing of the DNA bands indicates smaller DNA fragments with higher electrophoretic mobility than control \(\lambda\) DNA.

We have demonstrated that patulin under conditions similar to those used for anthracyclins and benzoquinone antibiotics cleaves DNA \textit{in vitro}. Patulin has been shown to induce DNA strand breaks in FM3A and HeLa cells\(^{5,10}\) and in \textit{E. coli} cells\(^{10}\). Our results support the idea of an involvement of free radicals of oxygen and \(\text{H}_2\text{O}_2\) in the mechanism of the DNA cleavage by patulin. Such a mechanism has been already proposed for the antibiotics mentioned above\(^{12,13}\). We do not believe that in the cell cupric ions play an essential role as probably the concentration of free cupric ions is too low. We would suggest that copper might be replaced by iron or another metal ion.

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References


