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Incorporation of Chlorine-36 into Ochratoxin A

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Incubation of *Aspergillus ochraceus* NRRL 3174 in a medium containing $Na^{36}Cl$ incorporated ^{36}Cl into ochratoxin A. The highest incorporation (0.75 and 0.70%, respectively) was obtained when the radioactive chloride was added to the medium by the 2nd or 3rd day of incubation.

Ochratoxin A is a toxic metabolite produced by certain species of *Aspergillus* (2, 7) and *Penicillium* (8). It comprises a 7-carboxy-5-chloro-3,4-dihydro-8-hydroxy-3-methyl-isocoumarin moiety linked to L-β-phenylalanine through the 7-carboxyl-group by an amide bond (Fig. 1).

In studies on ochratoxin A biosynthesis, the toxin has been labeled only on the phenylalanine moiety by unaltered incorporation of ^{14}C-phenylalanine (4, 5). The present report describes the incorporation of ^{36}Cl into ochratoxin A on the isocoumarin moiety.

MATERIALS AND METHODS

Organism. *Aspergillus ochraceus* Wilhelm NRRL 3174, obtained from C. W. Hesseltine, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., was used in the investigation.

Culture medium. The culture medium was similar to that of Ferreira (1) with a slight modification to reduce the chlorine content without affecting the production of ochratoxin A. One liter of the basal medium contained: KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; sucrose, 30 g; L-glutamic acid, 10 g; $Fe_2(SO_4)_3$, 18 mg; $ZnSO_4 \cdot 7H_2O$, 21.99 mg; $MnSO_4 \cdot H_2O$, 7.7 mg; $CuSO_4 \cdot 5H_2O$, 3.93 mg; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.52 mg. The *p*H was adjusted to 6.2 with KOH pellets. Flasks (500 ml) containing 100 ml of medium per flask were sterilized at 121 C for 15 min. Media were inoculated with conidia of *A. ochraceus* and incubated on a rotary shaker at 250 rev/min at 25 C in the dark. Before and at different time intervals during incubation, 10 μCi of $Na^{36}Cl$ solution, 2.3 mg of Cl in 0.5 ml, was added to appropriate flasks.

Extraction and isolation. After 6 days of incubation, both mycelia and culture filtrate were extracted by the method of Yamazaki et al. (9). Ochratoxin A was then isolated by preparative thin-layer chromatography (TLC). The plates were developed in benzene-acetic acid (3:1; reference 6); the product was homogeneous by analytical TLC in this solvent system.

Assay. The amounts of ochratoxin A were determined by spectrophotometry based on the molar absorbancy of 6,100 at 333 nm (3).

Determination of radioactivity. The crude extract and pure ochratoxin A were counted at infinite thinness with a thin-window (1.6 mg/cm²) Geiger counter. Samples of 10 μliters were dried down in the center of aluminum planchets and placed in a fixed position close to the window. The counting efficiency was 32.3%.

Scanning of radioactivity. The distribution of ^{36}Cl in the crude extract on the TLC plates was scanned with Actigraphy III (Nuclear-Chicago Corp., Des Plaines, Ill.) with the collimator slit set at 3 mm.

RESULTS AND DISCUSSION

The radioactivity was readily incorporated into ochratoxin A by *A. ochraceus* (Table 1). The ^{36}Cl added at day 2 or 3 of the cultures produced ochratoxin A with highest specific activi-

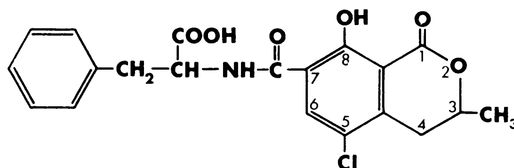

FIG. 1. *Structure of ochratoxin A.*

ties. This agrees well with the results of Ferreira (1) who found that the yield of ochratoxin A reached a maximum by the third day of the incubation. However, by the 5th day a small but still significant amount of ochratoxin A was formed in this experiment, perhaps representing a balance between synthesis and degradation of the toxin at this time.

The concentration of chloride used in the Ferreira medium was 7 mM, whereas in this experiment only 1.7 mM Cl^- was used. No significant decrease in ochratoxin A production was observed at this low Cl^- concentration (Table 2). A 10-μCi amount of ^{36}Cl, equivalent to 0.065 mmole of Cl^-, added to the medium (total Cl^-:

TABLE 1. *Incorporation of ^{36}Cl into ochratoxin A*[a]

Time of ^{36}Cl addition (days)	Total radio-activity of crude extract (μCi)	Total radio-activity of ochratoxin A (μCi)	Yield of ochratoxin A (mg)	Specific activity (mCi/mole)
0	0.67	0.58	10.6	22
1	0.69	0.60	10.5	23
2	0.77	0.75	11.2	27
3	0.71	0.70	9.9	28
4	0.59	0.55	10.8	20
5	0.09	0.08	10.5	3

[a] Ten microcuries of Na^{36}Cl was added to cultures of various ages. All of the cultures were harvested after 6 days of incubation. Values are averages of two experiments.

TABLE 2. *Effect of chloride content on the production of ochratoxin A*[a]

Chloride concn in medium (mM)	Yield of ochratoxin A (mg)
7.0	10.6
2.4	10.1
1.7	10.5
0	0.2

[a] Values are averages of two experiments.

0.24 mmole) had no effect on the amount of ochratoxin A produced.

The radioactivity in the crude extracts was found exclusively in the ochratoxin A fraction. A typical thin-layer chromatogram photographed in ultraviolet light, together with a radioscan of the plate, is shown in Fig. 2. The position of the most prominent fluorescent, radioactive spot corresponds exactly with that of authentic ochratoxin A (R_F 0.54). No significant radioactivity could be detected in any of the other components of the sample chromatographed.

Since ochratoxin A labeled with ^{14}C in the phenylalanine moiety has been prepared (4, 5), the present work opens the way to preparing doubly labeled material, which would offer obvious advantages for studies of the metabolism and mechanism of action of this toxin.

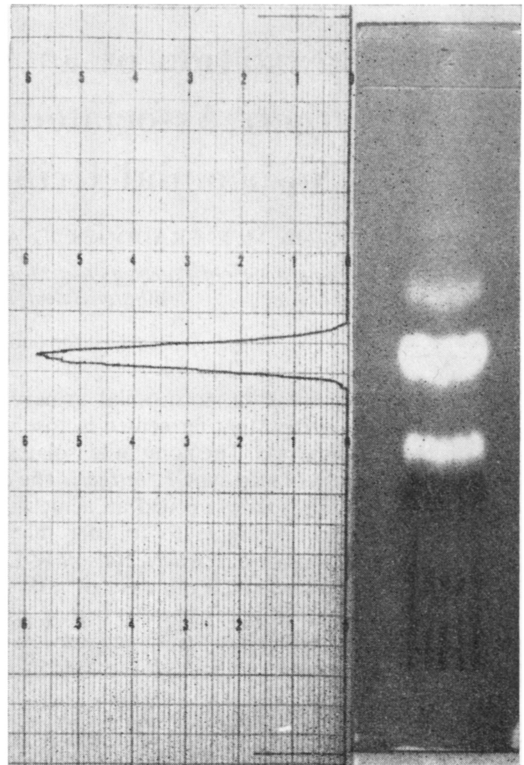

FIG. 2. *Thin-layer chromatography of the crude extract from the culture to which ^{36}Cl was added by the 2nd day of incubation. Each spot represents 1/1,000 part of the total extract. Left: radio scanning of the chromatoplate. Right: irradiation of the plate with long-wave ultraviolet light.*

ACKNOWLEDGMENTS

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