Preparation of $^{14}$C-Labeled Sterigmatocystin in Liquid Media

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$^{14}$C-labeled sterigmatocystin was prepared from surface cultures of *Aspergillus versicolor* A-18074 maintained in liquid media by multiple additions of [$1^{-14}$C]acetate to the cultures. The highest yield of 7.75 mg/10 ml was found with a sucrose-asparagine-ammonium medium in which more than 3% of the radioactivity of the added [$1^{-14}$C]acetate was recovered in the purified [ring-$^{14}$C] sterigmatocystin. The method offers an easy way to prepare $^{14}$C-labeled sterigmatocystin for studies of this mycotoxin.

Sterigmatocystin (ST), a biogenetic precursor of aflatoxin (AF) B$_1$ (6), is produced by several species of common fungi (5). Its carcinogenic effects on various experimental animals (9, 10), as well as its possible widespread occurrence in foodstuffs (14), has recently prompted the Food and Drug Administration to contract investigations of various aspects of this significant mycotoxin.

Preparation of ST from cultures of *Aspergillus versicolor* has been mentioned in several research reports concerning studies of the biosynthesis of ST and AF B$_1$ (3, 4, 12). However, detailed description specifically on the preparative techniques is not yet available. The present paper deals with preparation in several liquid media of $^{13}$C- and $^{14}$C-labeled ST to be used as analytical standards or in metabolic studies.

**MATERIALS AND METHODS**

**Organism and media.** *A. versicolor* A-18074, a strong ST producer obtained from the U.S. Department of Agriculture, Northern Regional Research Laboratory, Peoria, Ill., was used in the present experiments. A conidia suspension in 0.01% aqueous sodium lauryl sulfate containing approximately 10$^9$ spores/ml was used to inoculate the liquid media.

The low salt medium of Reddy et al. (11), containing as major constituents sucrose, asparagine, and ammonium sulfate, was used to grow the mold and produce ST. In some cultures, the nitrogen-free resting cell medium of Hsieh and Mateles (8) supplemented with glucose (30 g/liter) was used to replace the low salt medium at certain stages of incubation to be described below.

**Culture techniques.** Three techniques were tried. First, about 10$^5$ conidia were inoculated into 10 ml of low salt medium contained in each 50-ml conical flask, and the flasks were incubated in the dark at 29 C, without shaking, as surface cultures. Second, the low salt medium in the flasks of the surface cultures was replaced with the same volume of resting cell (RC) medium at the 4th day of incubation when ST synthesis in the mycelia started. Third, the 4-day-old mycelial mats were collected on cheesecloth and dispersed in RC medium with an electric blender, and the mycelial suspension was distributed into flasks and shaken at 120 rpm in a rotary shaker at 29 C. The cultures were incubated for a total period of 24 days for ST production unless otherwise noted.

To incorporate $^{14}$C-labeled acetate into ST, [$1^{-14}$C]acetate (New England Nuclear Corp., Boston Mass.; 59.5 Ci/mol) was added to the cultures after 4 days of incubation. Acetate was added at appropriate time intervals rather than all at once to maintain the acetate concentration below 10 mM. The concentrations of acetate in the medium were determined by monitoring the radioactivity in the medium.

**Purification of ST.** ST in the mycelia and spent medium was exhaustively extracted with acetone and ethyl acetate, respectively. The extracts were pooled, and the solvents were evaporated under reduced pressure to dryness at room temperature. The residues were dissolved in small amounts of chloroform and were purified by two thin-layer chromatography (TLC) systems in a series: ChromAR Sheet 500 (Mallinkrodt Chemical Works, St. Louis, Mo.) developed with benzene-acetone (96:4, vol/vol) and Adsorbosil-1 (Applied Science Lab., State College, Pa.) plates developed with chloroform-acetone-n-hexane (85:15:20, vol/vol/vol). The ST bands on plates were detected under the long-wavelength ultraviolet light. The purified ST was then recrystallized in a mixture of chloroform and isopropanol.

**Analyses.** The dry cell mass was measured by heating the washed mycelial mats at 110 C to a constant weight.

The concentration of ST in chloroform was calculated from the optical density of the chloroform solutions at 327 nm by using the extinction coefficient 16,220 (13). The ultraviolet spectrum of the product was obtained from a Beckman DK-2A ratio recording spectrophotometer to examine its purity as compared with the spectrum of ST purchased from Makor Chemicals Ltd., Jerusalem, Israel.

The radiochemical purity of $^{14}$C-labeled ST produced was examined on another TLC system: Brinkmann Silica Gel 60 precoated plate developed with
benzene-ethanol-water (85:15:1, vol/vol/vol), followed by determination of the radioactivity distribution on the plate by a Packard model 7201 radiochromatogram scanner and by autoradiography on X-ray films (Eastman Kodak Co., Rochester, N.Y.). The radioactivity was measured with a Packard Tri-Carb model 2405 liquid scintillation spectrometer.

RESULTS

Purity of 14C-labeled ST. The characteristic peaks and the relative peak heights in the ultraviolet spectrum of the recrystallized product in ethanol were the same as those reported in the literature (1) for standard ST. When co-chromatographed with the standard on the TLC system, Brinkmann Silica Gel 60 precoated plate developed in benzene-ethanol-water, the product gave only one compact spot with an $R_f$ matching that of the standard. The autoradiograph of the developed TLC plate indicated a concentration of all the detectable radioactivity within the absorbent zone containing the product. The distribution of the radioactivity on the developed TLC plate as determined by the Packard radioscaner is shown in Fig. 1. The additional TLC system also indicated that the crystalline 14C-labeled ST had a constant specific activity.

Production in different cultures. The three culture techniques described in Materials and Methods, all potentially useful in the incorporation of 14C-labeled acetate into ST, were compared for the yields of ST (Table 1). The standing culture with its medium not disturbed produced the highest amount of ST. The same culture with its medium replaced by the nitrogen-free resting cell medium containing comparable amounts of glucose produced slightly less ST. The shaking suspension of the dispersed mycelia in the resting cell medium incubated as a submerged culture yielded very little ST. Therefore, the standing culture was chosen to produce 14C-labeled ST for its high yield and simplicity.

The time course of the fungal growth and ST production in the standing culture is shown in Fig. 2. The mycelial mat was formed to cover the surface of the medium in 4 days, and the mycelium stopped growing in 5 days. ST was detectable in the mycelium in 4 days; thereafter, ST was produced at a constant rate for about 10 days. During the first 5 days of incubation, the pH of the medium dropped from 5.8 to 4.2 and then increased to 7.8 and stayed there. The rise in pH seemed to be associated with the ST production only in the low salt medium. It was not observed in the cultures maintained in the resting cell medium. The time course of ST production indicated that the high synthetic activity in the mycelium prevailed from the 5th day of incubation on for about 10 days, during which a high efficiency of 14C-labeled acetate incorporation into ST could be expected.

Incorporation of 14C-labeled acetate into

TABLE 1. Production of ST by A. versicolor using different culture techniques*  

| Culture† technique | ST (mg) | Dry cell mass (g) | Specific productivity (mg of ST/g of DCM*) |
|--------------------|---------|-------------------|------------------------------------------|
| I                  | 6.06    | 0.253             | 23.94                                    |
| II                 | 5.59    | 0.224             | 24.96                                    |
| III                | 0.48    | 0.229             | 2.09                                     |

* All runs were made in 50-ml conical flasks each containing 10 ml of medium.
† I, Standing cultures in LS medium; II, standing cultures in RC medium; III, shaking mycelial suspension in RC medium.
‡ DCM, Dry cell mass.
ST. In the incorporation of $^{14}$C-labeled acetate into AF, it was found that acetate concentrations exceeding 10 mM were detrimental to the incorporation efficiency (8); therefore, in the present preparation of $^{14}$C-labeled ST acetate concentrations lower than 10 mM were used. Determination of acetate uptake by the standing culture indicated that 10 mM of acetate disappeared from the medium in about 10 h. Therefore, during the 10 days of the synthetically active period, 20 additions of $^{14}$C-labeled acetate can be made at appropriate intervals to increase the specific activity of the product without affecting the incorporation efficiency. The results of some trial runs are summarized in Table 2. Comparing the radioactivity recovered from the products of runs 2 and 3, it is evident that the total radioactivity incorporated was proportional to the number of precursor additions, and the incorporation efficiency (more than 3%) remained unaffected.

**DISCUSSION**

Experimental evidence is now available for the biogenetic relationship between ST and AF (1, 6, 12). Therefore it is reasonable to believe that the two metabolites share the same biosynthetic pathway and are subject to similar metabolic regulations. It has been shown that the biosynthesis of AF occurs extramitochondrially in the fungal cells and that acetyl coenzyme A derived from exogenous acetate rather than glucose is preferentially used as the precursor because of the permeability barrier of the mitochondrial membrane (7). Therefore, it follows that acetate is likely also a preferential precursor of ST and that the specific activity of $^{14}$C-labeled ST is determined by the concentration of $^{14}$C-labeled acetate relative to the concentrations of other carbon sources in the culture medium.

Since one molecule of ST is formed from 9 acetate units (4, 12), it is theoretically possible to synthesize $^{14}$C-labeled ST with a specific activity nine times that of the $^{14}$C-labeled acetate used. The low specific activity of the $^{14}$C-labeled ST produced in the present study is a result of the low concentration of $^{14}$C-labeled acetate relative to the concentrations of sucrose and asparagine in the medium. Especially asparagine has been found to behave very much like acetate in terms of rapid uptake by the mycelium and efficient incorporation into AF (our unpublished data); therefore, it effectively diluted $^{14}$C-labeled acetate during the label incorporation process. The specific activity of $^{14}$C-labeled ST can be increased simply by increasing the number of additions of $^{14}$C-labeled acetate and/or reducing the concentration of asparagine in the medium. The reduction of asparagine concentration, however, would most likely decrease the yield of ST. The specific activity of $^{14}$C-labeled ST can also be increased by shortening the incubation of the culture after the last acetate addition. In this case ST would be produced largely in the presence of $^{14}$C-labeled acetate, and more carbons in ST would be derived from $^{14}$C-labeled acetate.

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