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Muscarine: Isolation from Cultures of Clitocybe rivulosa

Abstract. *Muscarine has been isolated in a yield of 0.013 percent from mycelia of* Clitocybe rivulosa *grown in the laboratory on a medium supplemented with beer wort. Its reineckate and aurichloride derivatives were prepared.*

Muscarine has previously been isolated from the carpophores of species of *Inocybe, Amanita,* and *Clitocybe* (*1*); we have isolated it for the first time from the mycelium of a laboratory-grown culture of *Clitocybe rivulosa* (Pers. ex. Fr.) Kummer. Occurrence of the compound in natural carpophores has been reported on the basis of chromatographic data or biological activity; in one study muscarinic activity was found (*2*) in aqueous extracts of laboratory-grown *Inocybe rimosa.* We selected the species from among several of the genera *Amanita, Inocybe, Boletus,* and *Clitocybe* which were studied (*3*) to reveal their capacity to produce compounds with muscarinic activity (*4*) in the mycelium or broth of surface-grown cultures.

The culture (*5*), maintained on potato-dextrose agar, was grown in 1-liter Roux bottles on the surface of a medium (100 ml per bottle) composed of beer wort, 25 percent; mannitol, 2 percent; succinic acid, 1 percent; KH_2PO_4, 0.1 percent; $MgSO_4 \cdot 7\ H_2O$, 0.08 percent; $FeSO_4 \cdot 7\ H_2O$, 0.0013 percent; tap water; and NH_4OH solution to *p*H 5.2. Incubation was at 24° ± 1°C for 6 to 8 weeks. The pooled mycelial mats (491 g wet weight), extracted with 0.5 percent acetic acid (4 liters), yielded muscarine (0.013 percent, dry-weight basis) obtained as the reineckate (38 mg) by the following procedure.

The acid extract, concentrated to 300 ml *in vacuo,* was desalted by adding 6 volumes of ethanol, filtering, and reconcentrating. The desalting process was repeated twice. The final syrupy concentrate (15 ml) was diluted with water to 50 ml and extracted three times with equal volumes of *n*-butanol. The aqueous layer was concentrated to 10 ml and adsorbed on 9 g of a mixture of neutral alumina-celite (1 : 2), and the powder was packed above a column, 2 by 4.3 cm, of dry-packed, grade 1 neutral alumina. Elution with benzene-ethanol (2 : 1) gave fractions that contained muscarine, shown by thin-layer chromatography analysis with modified Dragendorff's reagent. Concentrate of combined fractions, about 3.5 ml, was purified by paper electrophoresis (4 by 41 cm Whatman No. 1 paper; 0.2*M* acetate buffer, *p*H 4.8 ± 0.1; 0.5 ma/cm; 7.5 hr) by applying 125 to 150 μl on the anode side of each strip. The strip area containing muscarine, as revealed by controls, was extracted with methanol in a Soxhlet apparatus. The methanolic extract was taken to dryness, the residue was dissolved in water and sufficient NaOH solution to bring the *p*H to about 12, and the solution (about 4 ml) was treated with ammonium reineckate solution (13 percent in methanol) to give muscarine reineckate. Recrystallization from acetone-isopropanol gave the derivative with a melting point of 178° to 180°C corrected; undepressed in admixture with a reference sample (*6*). The infrared spectra of the isolated derivative and of the reference sample were identical. Muscarine chloride was generated from the reineckate by passage of a solution through the chloride form of AG 1-X4 anionic exchange resin. Muscarine gold chloride prepared from the salt gave a melting point of 118° to 120°C corrected; reported (*7*) 118° to 121°C.

These data provide essential evidence that muscarine is biosynthesized by *Clitocybe rivulosa* in artificial media supplemented with beer wort and that the fruiting body of the fungus is not essential to this capacity.

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Collagen-Coated Cellulose Sponge: Three-Dimensional Matrix for Tissue Culture of Walker Tumor 256

Abstract. *Three-dimensional growth of large populations of cells in vitro has been observed in the interstices of a matrix consisting of collagen-coated cellulose sponge. The growth of Walker tumor 256 in this composite matrix is compared with that found in a matrix composed of either cellulose sponge alone or collagen sponge alone. The composite matrix is superior to either one. Collagen-coated cellulose sponge may provide a simple tool for the study of social interaction of cells in the formation of organized elementary tissue structures.*

Many investigators now use, as an optimal substrate for growing cells as monolayers, a glass surface coated with a thin film of collagen rather than bare glass. This development had its beginning in the work of Ehrmann and Gey, who found that several kinds of cells adhere to and grow better on collagen-coated glass than they do on bare glass (*1*). Study of the three-dimensional structure of tissues in vitro has been conducted with methods of organ culture, rotation mediated aggregation, and with sponge-matrix tissue culture (see *2*).

Although we have used matrices of cellulose sponge and plasma clot in a number of studies, we have encoun-