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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-

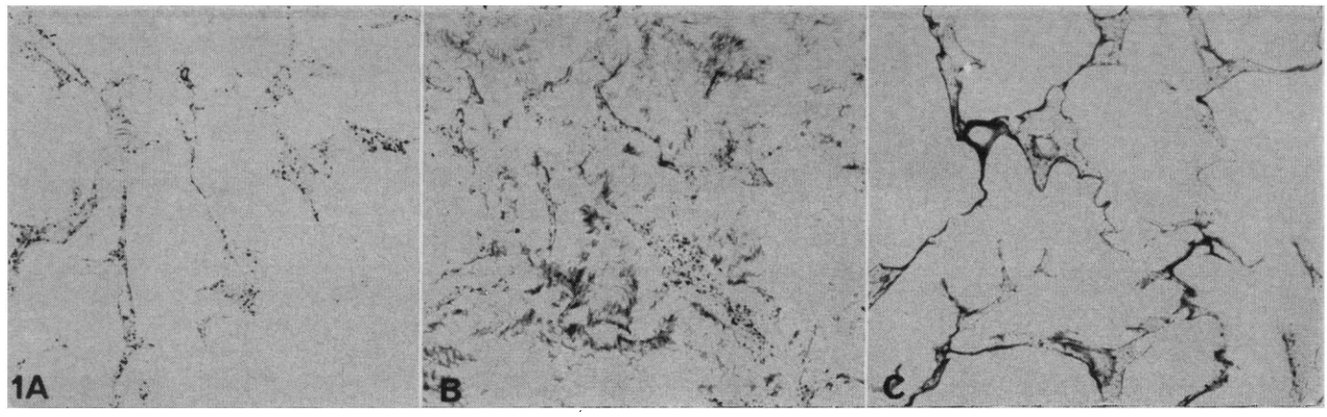

Fig. 1. Microscopic sections of stages in the preparation of collagen-coated cellulose sponge. Bouin's fixation, Masson trichrome stain (× 140). (A) Fine-pore cellulose sponge (DuPont) before impregnation with collagen. (B) Sponge fixed immediately after thorough impregnation with a liquid dispersion of collagen fibers. Fine fibers of collagen are seen throughout the interstices. (C) Sponge fixed 12 hours after impregnation. The dispersion medium, 50 percent methyl alcohol, has evaporated, and the collagen fibers have coalesced, becoming firmly attached to the surfaces of the cellulose trabeculae.

tered several limitations in this method (*3*). Tissue grows more densely in the fluid-bathed regions of the clot-sponge complex, rather than growing in a uniform distribution of proliferating cells throughout the sponge. More serious is the difficulty encountered with tissues that are highly lytic to clots of chick plasma. The outstanding ones among these are epithelial tissues, including carcinomas. When lysis of the clot occurs, most cell types that have grown out on the plasma clot do not adhere to the cellulose, but instead are washed into the medium during the course of continuous gentle agitation in the incubator.

The culture of tissues in a cellulose sponge coated with collagen, and without a plasma clot, seemed to be the answer to both problems. Fibrinolysis would not be a factor, and the interstices of the sponge would be patent rather than filled with a fibrin gel. We have prepared cultures on collagen-coated cellulose sponge and have compared the growth of Walker tumor 256 in collagen-coated cellulose sponge and in cellulose sponge alone.

The fibrillary collagen is prepared by Ethicon as follows. A portion of the deep flexor tendon of cattle is cleaned of fat, superficial noncollagenous protein, and other extraneous matter. It is sliced frozen on an electric meat slicer, perpendicular to the longitudinal axis of the tendons, at a thickness of about 0.4 mm. The slices are gently agitated for 1 hour at 35°C in a solution containing commercial ficin. They are then washed for 30 minutes with distilled water, after which time the enzyme is deactivated. Further extraction of polysaccharides and contaminant proteins is achieved by two successive 1-hour extractions in 1 percent neutral salt. The wet slices are added to a mixture of equal parts of methanol and water containing 2 percent cyanoacetic acid. The mixture is agitated for 30 minutes at 4°C and then homogenized by being pumped through orifices.

The cellulose sponge is prepared as 1 mm-thick slices and washed as previously described (*4*). The collagen dispersion, a viscous opalescent fluid, is dispensed with a tissue-culture pipette. A few drops of the dispersion (containing approximately 1 percent collagen in fibrillary form dispersed in the methanol-water base containing 2 percent cyanoacetic acid) is placed on a

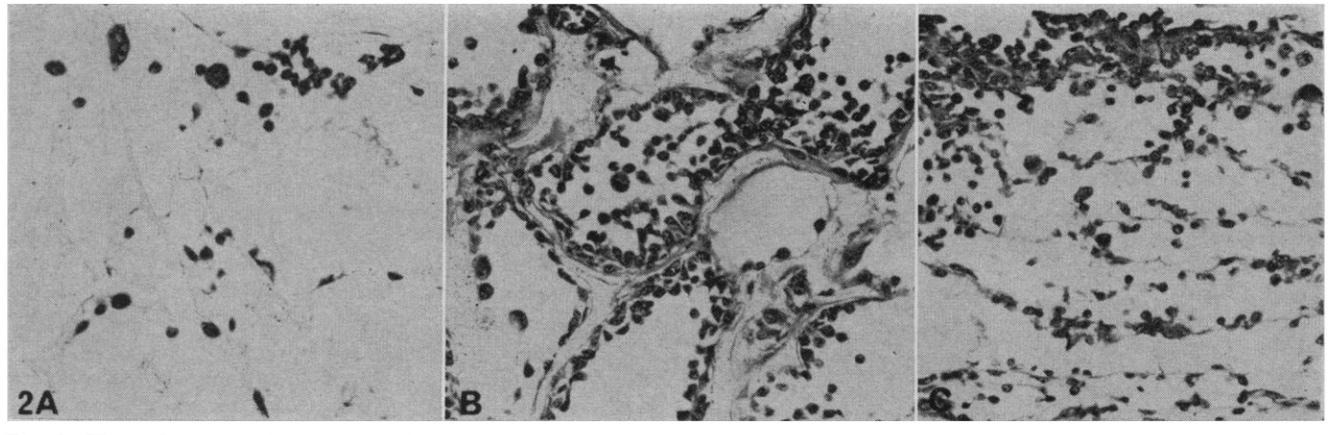

Fig. 2. Three fields showing the adhesion and growth of Walker tumor 256, 6 days after cultivation on cellulose sponge, on collagen-coated cellulose sponge, and on collagen sponge. Bouin's fixation, Masson trichrome stain (× 140). (A) Cellulose sponge alone. (No plasma clot has been used in this study.) The population is scanty and most of the cells are not adherent to the cellulose sponge. (B) Collagen-coated cellulose sponge. There is a large population of cells in the alveolae of the matrix. Many cells appear to be firmly attached to the collagen surfaces lining the spaces. Others are suspended in the alveolae. (C) Collagen sponge alone. The medium-bathed surface of the sponge is at the upper part of the figure where there is a dense population of cells. A smaller population is found below the surface.

slice of dry cellulose sponge, and the mixture is kneaded thoroughly into the sponge with a pair of forceps. A section of uncoated cellulose sponge is seen in Fig. 1A. The appearance of a sponge immediately after impregnation with collagen is seen in Fig. 1B. The sponges are placed in petri dishes and dried overnight at 38°C. As the collagen-dispersing medium evaporates, the fibers coalesce, deposit on, and adhere firmly to the cellulose trabeculae as thin sheets of collagen (Fig. 1C). The coated sponges are then washed for 12 to 24 hours in 50 percent methanol containing 0.5 percent ammonium hydroxide, followed by two 1-hour washes in sterile distilled water. The sponges are washed for 1 hour in Hanks's balanced salt solution and then stored in fresh Hanks's solution, *p*H 6.8, at 4°C until ready for use.

The difference in growth of Walker tumor 256 has been studied in three different matrices—cellulose sponge only, collagen sponge (Ethicon) only, and collagen-coated cellulose sponge.

A dense population of the tissue-culture line of Walker tumor 256 (Pgh No. 63–79) growing on the flat surface of a glass culture tube (*5*) was scraped loose in 9 ml of fresh medium (30 percent calf serum and Eagle's minimal essential medium, in Hanks's balanced salt solution). The suspension was divided equally among three large, flat culture tubes, each containing four pieces of one of the types of sponge. Each piece of sponge measured 12 by 6 by 1 mm. The suspension of cells was thoroughly irrigated through each piece of sponge. The recipient tubes were stoppered and incubated at 35°C on a stationary rack for 1 day. Subsequently they were placed on a rocking platform in the incubator and fed appropriate, increasing volumes of nutrient depending on the changes in *p*H produced in the medium by the growing cells.

In the first experiment one sponge in each group was fixed in Bouin's after 2 days, and the remaining three were fixed after 6 days. In the second experiment prepared in the same way, two sponges in each group were fixed after 8 days and two after 13 days.

Both experiments gave similar results. Adhesion of the cells on the bare cellulose trabeculae was meager, whereas collagen-matrix surfaces—either collagen-coated cellulose or collagen sponge alone—were covered with adherent proliferating cells. The difference was striking in less than a week. The results observed in the sponges fixed only 6 days after inoculation are illustrated in Fig. 2, A–C. As growth proceeded in sponges made of collagen alone, the mass of each sponge progressively decreased to less than half of its original size during a 2-week period of cultivation. Histopathologic examination of such older cultures revealed the surface of the sponge, the part in immediate contact with the medium to be covered with viable cells, but the remainder of the matrix consisted of frayed, collapsed trabeculae of collagen and of necrotic cells. In contrast, during the same period the collagen-coated cellulose sponge retained its shape, including the pattern of its open alveolae. Medium permeated the sponge through these connected spaces permitting vigorous growth throughout the matrix.

Collagen-coated cellulose sponge as a matrix for supporting three-dimensional growth in vitro eliminated the major problems of the combined plasma clot and sponge and of collagen sponge alone. It retains the best features of each. The cellulose matrix allows the interstices in the sponge to remain open. The lining of the trabeculae by collagen provides a surface on which a variety of cells adhere. In the interstices of the sponge, lacunae of varying sizes provide foci where groups of cells may more readily retain in their microenvironment the products of their metabolism.

Using several kinds of inocula we have recently observed that collagen-coated cellulose sponge provides an excellent matrix for three-dimensional growth. These include suspensions of HeLa cells and of rodent ascites hepatomas, as well as explants of chick embryonic heart and liver.

This method facilitates the study of organized growth of cell populations under conditions that permit the correlation of observations in vitro with histopathologic examination of material in vivo.

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D-Glucose: Preferential Binding to Brush Borders Disrupted with Tris(hydroxymethyl)aminomethane

Abstract. *The actively transported sugar* D-*glucose binds to brush borders disrupted with tris(hydroxymethyl)aminomethane in preference to* D-*mannose and* L-*glucose, which are not actively transported. This preferential binding of* D-*glucose is not dependent on either added* Na^+ *or adenosine triphosphatase activity stimulated by* Na^+ *with* K^+ *and* Mg^{2+}, *but it is temperature-dependent and is completely inhibited by 0.1 millimolar phlorizin and 1 millimolar mercuric chloride.*

It is currently thought that the mechanism of active transport of sugar by the small intestine comprises two steps: (i) an energy-independent, Na^+-dependent binding of the sugar to a "carrier" and (ii) an energy- and Na^+-dependent process which results in the dissociation of the "sugar-Na^+-carrier" complex, causing the sugar to be accumulated against its own concentration gradient (*1*). Furthermore, it has been proposed that a polyvalent or polyfunctional "carrier" with multiple binding sites may represent an integral portion of the membranes of the microvilli or brush borders of the mucosal cell (*2*) which is responsible for active sugar transport (*3*), rather than a specialized molecule shuttling back and forth within the membrane barrier. Phlorizin can competitively inhibit sugar transport, perhaps by virtue of its greater affinity for a site on the "carrier" within the brush-border membrane (*4*). In addition, data of autoradiographic studies suggest that phlorizin is bound to the mucosal microvilli and does not penetrate the mucosal cell (*5*).

We determined if the actively trans-