An Investigation of the Culture, Constituents, and Physiological Activity of *Panaeolus campanulatus**

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Wild-growing and cultivated carpophores of *Panaeolus campanulatus* were found to contain serotonin, 5-hydroxytryptophan, tryptophan, urea, and citrulline, but none of these compounds was present in the mycelium or nutrient medium obtained from cultures of the organism. Five additional indole derivatives were detected in the carpophores. These remain unidentified although they were shown not to be psilocybine, tryptamine, or N,N-dimethyltryptamine. An extract of the mushroom was found to exhibit parasympathomimetic activity which was muscarinic in nature. Choline was identified chromatographically, but muscarine, which was shown physiologically to exist in minute amounts in an extract, could not be detected by the methods employed. After physiological tests in small animals revealed that the mushroom was relatively nontoxic, a quantity was ingested by one of the investigators to test its reported hallucinogenic activity. No effects were noted, and it is concluded that this fungus does not produce cerebral mycetism nor is it a dangerously poisonous mushroom.

CONSIDERABLE CONFUSION centers about the black-spored agaric commonly referred to as *Panaeolus campanulatus* (Fr.) Quélet. Even its proper scientific name is in dispute. Singer and Smith (1) have concluded that if the species is considered conspecific with *Panaeolus sphinctrinus* (Fr.) Quélet, it should be known under that name, and if not, as *Panaeolus linnaenus* Imai. For reasons which will be advanced later in this paper it would appear that the latter name should be adopted.

The physiological activity of *P. campanulatus* is also controversial. Several cases of cerebral mycetism produced by members of the genus have been reviewed by Heim (2). Of these cases one, which was reported in 1816, was attributed to *Agaricus* (*Panaeolus*) *campanulatus*, but at this time it cannot be ascertained if the species identification was correct. Singer (3) has pointed out that members of this genus are not determinable by the layman and only with difficulty by the specialist.

Availability of a quantity of authentic *Panaeolus campanulatus* prompted an investigation of some of the chemical and physiological properties of the mushroom in an attempt to clarify some of these problems. Since the fungus had been shown previously (4) to contain a number of indole derivatives, including serotonin, a pure culture of the organism was prepared and investigated with respect to its ability to biosynthesize derivatives of the indole nucleus.

EXPERIMENTAL

Collection of Mushrooms.—Specimens of the mushroom were collected during the spring of 1958 near Olympia, Washington and were identified as *Panaeolus campanulatus* (Fr.) Quélet by Prof. D. E. Stuntz, Department of Botany, University of Washington. A culture was prepared from one of the fresh carpophores by carefully breaking open the pileus and removing a bit of the sterile, internal tissue with a mycologist's spade. The tissue fragment was transferred to a culture tube containing a slant of dung agar medium (5) where it began to produce typical mycelial strands within a few days. Some of the remaining carpophores were dried in a circulating-air oven at 50°, others were preserved by immersion in 95% ethanol.

Cultivation and Extraction.—In order to determine if the organism produced serotonin and related indole compounds in its mycelial state, four Roux-type culture bottles, each containing 125 ml. of the liquid medium of Heim, *et al.* (6), modified by the substitution of 4.5% malt extract for the beer wort, were prepared. The flasks were inoculated and placed in a constant temperature cabinet, which admitted subdued artificial light through its glass doors, and maintained at 25 ± 1° for a period of thirty days. Under these conditions the fungus underwent moderately rapid mycelial development, but no fruiting bodies were produced. At the conclusion of the incubation period, the mycelial mats were removed from the flasks, dried in a circulating-air oven at 50°, and weighed. The average dry weight of mycelium was 1.43 Gm. per flask.

The dried mycelium was reduced to a No. 60 powder in a Wiley laboratory mill, and a 3-Gm. quantity was placed in a test tube together with 15 ml. of 70% ethanol and agitated vigorously on a mechanical shaker for two hours. At the conclusion of this period the tube was centrifuged, and the extract decanted and allowed to evaporate to dryness at room temperature. The residue was redissolved in 2 ml. of 70% ethanol, and 60-μl. quantities of this solution were subjected to chromatographic analysis according to the procedure subsequently described.

The growth medium was decanted from the flasks, measured, and found to average 82 ml. per flask. The culture liquids of all the flasks were combined and reduced to dryness under reduced pressure in a Flash-Evaporator at 50°. This residue was then re-extracted with 100 ml. of 70% ethanol and the resulting extract filtered and evaporated as before to a vol-

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ume of 8 ml. Forty-microliter portions of this extract were then analyzed chromatographically.

To simulate more nearly in the laboratory the natural growth conditions of the fungus, a quantity of fresh horse dung, together with a little tap water, was placed in each of twelve 500-ml. Erlenmeyer flasks. After autoclaving, the flasks were inoculated and placed in the incubator at 25° where mycelial development took place and soon permeated the solid medium. After three weeks, five of the flasks were removed from the incubator and placed in a greenhouse where they were exposed to direct sunlight for several hours daily, as well as to diurnal temperature variations ranging between 15 and 35°. In less than two weeks normal carpophore development was noted in some of the flasks, and within four weeks all five showed fruiting body formation. These mushrooms were normal in every respect with the exception of slightly elongated stems (see Fig. 1). They were harvested periodically, counted, dried, and weighed, with the results recorded in Table I. The flasks continued to produce during a total period of fifteen weeks or twelve weeks after placement in the greenhouse.

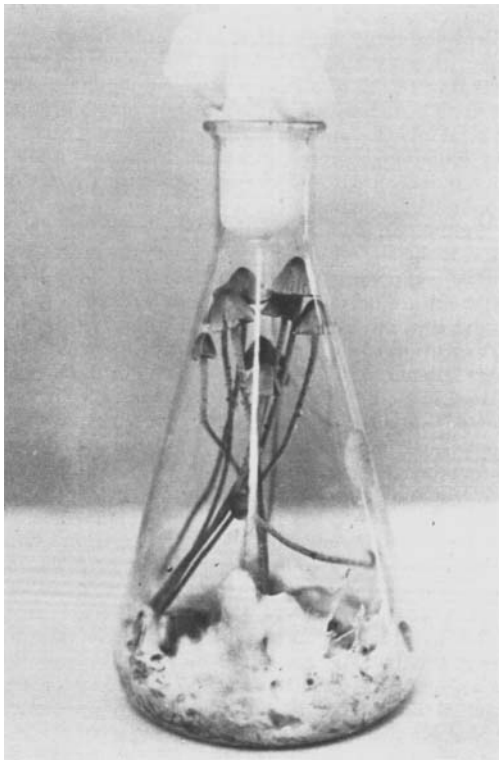

Fig. 1.—Cultivated carpophores of *Panaeolus campanulatus* in 500-ml. Erlenmeyer flask.

Of the seven flasks remaining in the incubator, none showed carpophore formation nine weeks after inoculation. In order to determine if the production of fruiting bodies was dependent on sunlight or was influenced merely by the temperature fluctuations, six of these remaining flasks were taken at the end of nine weeks and placed in the greenhouse; three in direct sunlight and three in a light-tight box. Two of the three flasks placed in the sunlight developed

TABLE I.—PRODUCTION OF CARPOPHORES OF *Panaeolus campanulatus* IN STERILE CULTURE

Flask No.	No. Carpophores	Fresh Wt., Gm.
1st Collection (6th Week of Growth)		
1	6	0.72
2	7	0.96
3	5	0.48
4	..	..
5	..	..
2nd Collection (8th Week of Growth)		
1	7	2.18
2	11	5.05
3	5	1.69
4	5	3.00
5	3	0.42
3rd Collection (12th Week of Growth)		
1	6	2.04
2	3	5.05
3	5	0.80
4	1	0.50
5	2	0.90
4th Collection (15th Week of Growth)		
1	4	3.00
2	1	0.67
3	10	4.55
4	2	1.30
5	3	1.03
Totals		
1	23	7.94
2	22	7.06
3	25	7.52
4	8	4.80
5	8	2.35
Av.	17	5.93

carpophores within two weeks. None of the cultures subjected to diurnal temperature variations in the absence of light produced carpophores during the six week period following their removal from the incubator. The single flask remaining in the incubator also failed to produce fruiting bodies during the entire course of the experiment.

Quantities of the dried carpophores, both cultivated and naturally occurring, were milled to a No. 60 powder and 0.5-Gm. quantities of each were extracted with 15 ml. of 70% ethanol as previously described. In this case, however, the final residues were each dissolved in 1 ml. of 70% ethanol and 40-μl. portions were subjected to chromatographic analysis.

Chromatographic Analysis.—Because of the large number of indole derivatives detected during the course of a preliminary investigation of *Panaeolus campanulatus* (4), it was necessary to utilize a two-dimensional paper chromatographic technique for satisfactory separation of all of these compounds. Investigation of a number of different acidic, neutral, and basic solvent systems revealed that effective separation was obtained when a mixture of *n*-propanol-1 *N* ammonia (5:1) was employed as the first wash liquid, followed by *n*-butanol-acetic acid-water (4:1:5) in the second direction. Sheets of Whatman No. 1 filter paper, $18^1/_4 \times 22^1/_2$ in., were utilized, and the direction of formation was ascending; time averaged twenty-two hours in each direction. The various extracts were spotted in replicate 10-μl. portions, each being dried before addition of the next, until the specified amount was applied. After

formation, the sheets were sprayed with 2% *p*-dimethylaminobenzaldehyde in 1 *N* hydrochloric acid (7) or with Pauly's reagent (8). Identification of the spots was based on comparison with colors and locations of reference compounds run as single compounds and in mixture with each other and with the various extracts. Average R_f values of the principal indole and urea derivatives, identified and unidentified, are recorded in Table II. Since these values vary slightly with the identity of the other components in the mixture and with concentration, these figures should be considered as indicative of the relative degree of separation achieved rather than absolute values.

TABLE II.—CHROMATOGRAPHIC DATA FROM EXTRACTS OF *Panaeolus campanulatus*

R_f[a] Values × 10²		Color Reaction With	Identity of Compound
Wild Carpophores	Cultured Carpophores	*p*-Dimethylaminobenzaldehyde	
3/15	2/13	Yellow	Citrulline
0/19	0/18	Bluish (faint)	?
6/25	4/24	Yellow	?
11/26	8/24	Purple	5-Hydroxytryptophan
17/39	17/35	Bluish (very faint)	?
20/46	20/46	Reddish violet	Tryptophan
17/78	17/79	Bluish (very faint)	?
28/79	30/79	Bluish	?
38/44	38/47	Yellow	Urea
53/49	49/51	Purple	Serotonin

[a] Expressed as: R_f obtained with *n*-propanol-ammonia/-R_f obtained with *n*-butanol-acetic acid-water.

After the pharmacological investigation had revealed the presence of a compound(s) exhibiting parasympathomimetic activity in this mushroom, quantities of an extract prepared in the manner described in the section on pharmacological evaluation were examined chromatographically for the presence of choline, acetyl choline, and muscarine. A solution of the extract was spotted on sheets of Whatman No. 1 filter paper and formed in an ascending direction for twenty-four hours with a wash liquid composed of butanol-ethanol-water (5:5:2). Kögl, *et al.* (9), report that in this solvent system choline exhibits an R_f of 0.37, acetylcholine 0.44, and muscarine 0.50. The chromatograms were evaluated with Thies and Reuther's reagent which Eugster (10) has reported is capable of detecting as little as 6 mcg. of muscarine.

When quantities of extract representing approximately 0.25 Gm. of fresh mushrooms were analyzed by this procedure, neither acetyl choline nor muscarine was observed, but a well-defined spot was noted with an R_f value averaging 0.36. This spot had the same R_f value as a control spot of known choline and did not separate from that reference compound in a mixed chromatogram. Although no muscarine was detected, calculation revealed the quantity found to be present by physiological methods in this amount of extract would be below the sensitivity of the chromatographic detection method employed.

Attempts to chromatograph quantities of the crude extract representing quantities of fresh mushrooms as large as 5 Gm. were made. On the basis of physiological tests, this quantity of plant material should contain approximately 65 mcg. of muscarine, a quantity readily detectable with Thies and Reuther's reagent. However, at this concentration, the relatively large amount of choline and other impurities in the extract produced a distortion of R_f values, and the presence of muscarine could not be verified.

Pharmacological Investigation.—For the preliminary physiological testing in small animals an extract was prepared from a 155-Gm. quantity of fresh *Panaeolus campanulatus* which had been preserved in 95% ethanol. The mushrooms and the alcohol (approximately 200 ml.) were homogenized in a Waring Blendor for two minutes and the cellular debris separated from the extract by filtration on a Büchner funnel. The marc was then returned to the Blendor vessel and re-extracted, first with 250 ml. of 70% ethanol for three minutes, then with two successive 325-ml. portions for five minutes each. The successively collected filtrates were combined and evaporated to a syrupy residue under reduced pressure in a Flash-Evaporator at 50°.

The residue was taken up in two successive 100-ml. portions of distilled water; the extracts combined, filtered, and again reduced to a solid residue in the Flash-Evaporator. The soluble portion of the residue was dissolved in a series of small volumes of distilled water (total 25 ml.) which were combined, evaporated, and the residue dried in a desiccator over calcium chloride. The solid extract weighing 2.84 Gm. and the dried marc from the alcoholic extractions (8.25 Gm.) were tested for gross physiological activity in rats.

Rats were previously unused twelve-week-old male animals of the Wistar strain, fasted overnight prior to testing. All injections were made intraperitoneally into the upper left quadrant of the abdomen after the rats had been weighed to the nearest whole gram. Dosage volume for all injections was 5 ml./Kg. Solutions of the extract were made utilizing distilled water, while solution-suspensions of the marc were prepared using 0.25% agar solution.

Injections of the marc produced no physiological effects up to 500 mg./Kg., which was the maximum dosage physically capable of being injected. At this peak dosage level, some ptosis, respiratory depression and abdominal tenderness were noted.

Injections of the extract were physiologically inert up to 1 Gm./Kg. at which level lacrimation, micturition, bloody tears, decrease in respiratory rate, increase in respiratory depth, and reduction of motor activity were noted. Maximal response was seen one to two hours after injection. These symptoms were all indicative of parasympathomimetic activity. No miosis was seen.

All animals receiving the extract and the marc were observed for two weeks after the injections. No delayed toxicity was observed.

Utilizing a modified Magnus technique (11), the extract was further screened using isolated rabbit intestine. Perfusion medium used was oxygenated Sollmann-Radamaeker's solution maintained at 37°; bath size was 100 ml. Rabbits were killed by a sharp blow at the back of the neck. Extract bath concentrations of 1:50,000 produced a submaximal increase of intestinal tone with little to no effect on peristaltic rate. A concentration of 1:2,500 had a depressant effect on tone and peristalsis.

In the absence of a sample of muscarine, methacholine chloride was used as a reference compound, as methacholine has been shown to be primarily muscarinic in action with little to no nicotinic activity (12). Concentrations of 1:100,000 methacholine chloride and 1:50,000 of the extract are roughly comparable qualitatively and quantitatively (see Figs. 2 and 3). A bath concentration of 1:10,000 atropine sulfate effectively blocked methacholine, but a bath concentration of 1:2,500 was required to comparably block the extract. The addition of the extract (1:5,000) to the bath containing methacholine partially reversed the atropine atony of the intestine refractory to methacholine. Raising the concentration of the extract from 1:50,000 to 1:4,500 partially reversed the atropine blockade of the extract. The ability of the extract to reverse the parasympatholytic effects of atropine is further illustrated in Fig. 4. Atropine sulfate, 1:10,000, effectively blocked methacholine chloride response at bath concentrations of 1:33,000, but was reversed by 1:2,500 concentration of the extract. Further addition of atropine, in turn, reversed the stimulation of the extract, indicating a competitive inhibition between these two agents. It has been noted that if muscarine and atropine are administered concurrently or successively, their respective quantities determine which effect predominates, although atropine is the stronger of the two (13).

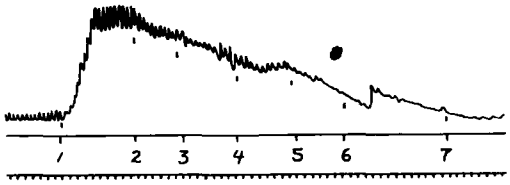

Fig. 2.—Additions to 100-ml. bath. *1*, 2 mg. extract; *2*, 10 mg. atropine sulfate; *3*, 10 mg. atropine sulfate; *4*, 10 mg. atropine sulfate; *5*, 10 mg. atropine sulfate; *6*, 20 mg. extract; *7*, 200 mg. extract.

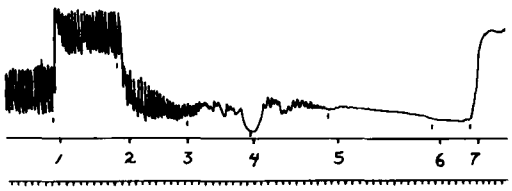

Fig. 3.—Additions to 100 ml. bath. *1*, 1 mg. methacholine chloride; *2*, 10 mg. atropine sulfate; *3*, 2 mg. methacholine chloride; *4*, 20 mg. extract; *5*, 2 mg. methacholine chloride; *6*, 2 mg. methacholine chloride; *7*, 10 mg. barium chloride.

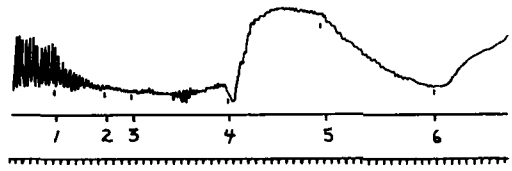

Fig. 4.—Additions to 100 ml. bath. *1*, 10 mg. atropine sulfate; *2*, 1 mg. methacholine chloride; *3*, 2 mg. methacholine chloride; *4*, 40 mg. extract; *5*, 20 mg. atropine sulfate; *6*, 10 mg. barium chloride.

Qualitatively the extract exhibits parasympathomimetic activity that is muscarinic in nature. Similar techniques utilizing isolated rabbit intestine have shown that muscarine produced submaximal effects at a bath concentration of 1:67,000,000 (14). Assuming that the activity of this extract is due to muscarine alone, the content of this extract would be approximately 0.07% and that of the fresh mushrooms approximately 0.0013%.

After its detection by chromatographic analysis, choline chloride was tested on isolated rabbit intestine. Bath concentrations of 1:100 produced submaximal effects comparable to those seen previously with methacholine and the extract (see Fig. 5). Acetylcholine chloride was also tested for reference purposes and produced submaximal effects at a concentration of 1:100,000. Complete blockade of choline and acetylcholine was effected by 1:10,000 concentrations of atropine sulfate. Further additions of choline and acetylcholine did not reverse the atropine atony. Assuming that the activity of the extract is due solely to choline, the choline content was calculated to be 50,000%, an impossible figure. The collected data indicate, therefore, that while the choline found in the extract contributed to the parasympathomimetic effect, there was a more powerful agent also present. Assuming that the major portion of the extract is choline, and that the remaining parasympathomimetic activity is due solely to muscarine, calculations still indicate about 0.07% of muscarine in the extract. The gross parasympathomimetic effects seen in the intact rat dosed with 1.0 Gm./Kg. of the extract cannot be produced by dosage levels of choline that could be physically present.

After the preliminary tests in rats had indicated that *Panaeolus campanulatus* was relatively nontoxic but possessed a certain amount of physiological activity, it was considered necessary to test the activity of the mushroom in man, since drugs influencing the higher brain centers in human beings do not always demonstrate their typical effects in lower animals. Six dried carpophores of the mushroom (dry weight, 0.8 Gm.) were ingested by one of us (V. E. T., Jr). Initially, two of the mushrooms were thoroughly masticated and swallowed. When no effects were observed after thirty minutes, four more carpophores were ingested. The mushrooms proved to be nearly tasteless at first but developed a slightly acrid, mucilaginous taste on chewing, which was considered to be somewhat unpleasant. No physiological effects of any kind were observed following the ingestion of this quantity of the mushroom.

RESULTS AND CONCLUSIONS

No qualitative differences were detected chromatographically in the composition of the wild and the cultivated carpophores of *Panaeolus campanulatus*. Serotonin, 5-hydroxytryptophan, tryptophan, urea, and citrulline were identified as constituents of both types of fruiting bodies, but none of these compounds was detected in the mycelium or the medium obtained from mycelial cultures of the organism. Identification of these compounds was based on R_f values and color reactions with *p*-dimethylaminobenzaldehyde. In

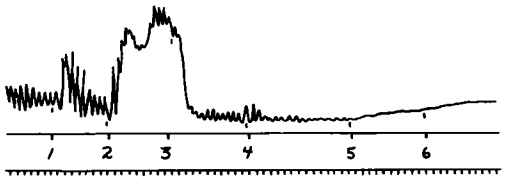

Fig. 5.—Additions to 100 ml. bath. *1*, 100 mg. choline chloride; *2*, 900 mg. choline chloride; *3*, 10 mg. atropine sulfate; *4*, 1,000 mg. choline chloride; *5*, 1,000 mg. choline chloride; *6*, 1,000 mg. choline chloride.

addition, the serotonin and 5-hydroxytryptophan exhibited characteristic pinkish colors after being subjected to Pauly's reaction.

The occurrence of 5-hydroxytryptophan is not surprising in view of the presence of serotonin in the mushroom and the fact that 5-hydroxytryptophan has been shown to serve as a precursor to serotonin, at least in animal tissue (15). The presence of both compounds here is indicative of a similar biosynthetic pathway in this fungus.

On the basis of the typical color reactions with *p*-dimethylaminobenzaldehyde, it was concluded that five additional unidentified indole derivatives and one other unidentified urea derivative were present in the carpophores. Comparison with the color reactions and R_f values of reference compounds indicated that none of these unidentified indole derivatives was psilocybine[1] (R_f 0/0.27), tryptamine (R_f 0.78/0.67), or N,N-dimethyltryptamine[2] (R_f 0.93/0.69). Psilocybine was not detected in the carpophores of *Panaeolus campanulatus* in spite of the recent report by Heim and Wasson (16) of its isolation from *Panaeolus sphinctrinus*. This would indicate that *Panaeolus campanulatus* and *Panaeolus sphinctrinus* are not conspecific and argues for the designation of the former species as *Panaeolus linnaenus* Imai.

The absence of indole derivatives from the mycelial cultures of the organism is particularly interesting in view of the report of Singer and Smith (1) that *Panaeolus subalteatus* (*venenosus*) produces an active hallucinogenic principle, which is presumably an indole derivative, in its mycelium. This, too, is indicative of certain differences in the metabolism of various indole compounds in different species of the genus *Panaeolus*. Although some species may contain hallucinogenic agents, *Panaeolus campanulatus* does not, and it should cease to be designated as a mushroom capable of producing cerebral mycetism.

An extract of the fungus did exhibit parasympathomimetic activity which was muscarinic in nature. Chromatographic analysis revealed the presence of choline in the extract, but the occurrence of muscarine could not be confirmed. On the basis of the physiological tests, it is estimated that approximately 0.0013% of the fresh weight of the mushrooms corresponds to muscarine; slightly more than the 0.0003% of this compound shown to exist in *Amanita muscaria* (10). This low level of the material in the mushroom no doubt accounts for the inability to verify its presence by chromatographic analysis of a crude extract.

Since the fatal oral dose of muscarine in the adult human being varies between 0.3 and 0.5 Gm. (17), a quantity equivalent to more than 23 Kg. of fresh *Panaeolus campanulatus*, it must be concluded that the toxicity of this mushroom is extremely low. Although it probably will never serve as an important comestible because of its relatively small size and unpleasant taste, *Panaeolus campanulatus* should not be classified as a dangerously poisonous mushroom.

[1] Reference compound supplied through the courtesy of Dr. A. Hofmann, Sandoz A. G., Basel, Switzerland.

[2] Reference compound supplied through the courtesy of Dr. F. A. Hochstein, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

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