TABLE IV—ISOTOPE EXCHANGE FOR COMPOUNDS II

Compd.	Recovery, %	Exchange, %	Spec. Act., mc./mg.
IIa	10	15	5.22
b	70	62.8	17.58

established by (*a*) melting point and (*b*) TLC and a radiochromatogram of the strip (see Table IV).

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Keyphrases

Tumor localizing agents—synthesis
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Interrelationship of Phosphate Nutrition, Nitrogen Metabolism, and Accumulation of Key Secondary Metabolites in Saprophytic Cultures of *Psilocybe cubensis, Psilocybe cyanescens,* and *Panaeolus campanulatus*

By J. M. NEAL, R. G. BENEDICT, and L. R. BRADY

The three basidiomycetes were grown on rotary shakers in four nutrient media containing various amounts of phosphate to determine the relative effect of this nutrient on the trichloroacetic acid-soluble and -insoluble nitrogen metabolites and to detect possible correlation between the fungal free amino acid pool (soluble nitrogen) and accumulation of characteristic hydroxytryptamine derivatives. The species were selected to represent different patterns of metabolism in fruiting bodies and vegetative mycelia. Vegetative mycelium of *P. cubensis* was characterized by a relatively high soluble nitrogen component and by the capacity to accumulate psilocybin and psilocin under selected conditions. The closely related *P. cyanescens* was less responsive to variations in phosphate nutrient and lacked the capacity to accumulate appreciable quantities of soluble nitrogenous compounds or detectable quantities of key tryptamines. *P. campanulatus* grown in phosphate-rich media appeared to have an adequate free amino acid pool and to excrete some exocellular nitrogen metabolites during longer incubation periods; no 5-hydroxytryptamine derivatives were detected in cultures of this fungus.

OBSERVATIONS on the occurrence of various secondary metabolites in basidiomycetes have demonstrated the medicinal interest in certain of these fungi (1). However, extensive investigation or utilization of many of the empiric observations has been delayed by the lack of basic biologic knowledge which would permit manipulation of the basidiomycetes in the manner of antibiotic-producing actinomycetes or various commercially useful ascomycetes. Information on the occurrence of many basidiomycete metabolites suggests that fungal fruiting bodies and

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saprophytic mycelia frequently exhibit different metabolic capabilities. Such distinctive metabolism is reminiscent of the situation with *Claviceps* species where lysergic acid alkaloids were known to occur in the sclerotium or resting stage of the fungus as early as 1875, but successful production of such derivatives in saprophytic culture was not achieved until 1953 (2).

Mushrooms belonging to the related genera *Panaeolus* and *Psilocybe* are known to contain 5- and 4-hydroxyindole derivatives, respectively. Patterns of accumulation of these metabolites during different growth phases of these fungi reveal a good potential for comparative studies which may contribute to an understanding of basic metabolic interrelationships. Serotonin and 5-hydroxytryptophan accumulate in the fruiting bodies of various species of *Panaeolus* (3–6), but these 5-hydroxyindoles have never been detected in mycelial cultures (7). Psychedelic 4-hydroxytryptamines, usually psilocybin and psilocin, occur in the carpophores of at least 13 *Psilocybe* species (8–10). However, production of 4-hydroxyindoles in submerged culture has been reported for only *Psilocybe cubensis* (Earle) Sing. (11) and *P. baeocystis* Sing. and Smith (12), and no detectable quantities of these compounds were observed in mycelial cultures of other species such as *P. cyanescens* Wakefield (11) which have been investigated.

Presumably, genetic factors contribute either directly or indirectly to the observed differences in patterns of accumulation of indolylic amines in vegetative mycelium and carpophore tissues, but very little is known about the influence of various nutrients or other environmental factors on the accumulation of these compounds or about other biologic processes in these fungi. The relationship of phosphate nutrition to nitrogen metabolism and accumulation of secondary metabolites was selected as a logical focus for initial biologic studies. A reduced availability of phosphate in culture media has been associated with an increased accumulation of such secondary metabolites as clavine alkaloids by *Claviceps* strain 47A (13), chlortetracycline by *Streptomyces aureofaciens* Duggar (14), and volucrisporin by *Volucrispora aurantiaca* Haskins (15). Precursor moieties for clavine alkaloids, chlortetracycline, and volucrisporin are known to be derived, at least in part, from amino acids. The response pattern of phosphate deficiency on nitrogen metabolism in fungi is not well established, but phosphate deficiencies in angiosperms are associated generally with a large increase in free amino acid nitrogen in the cells and a corresponding decrease in protein nitrogen.

Panaeolus campanulatus (Fr.) Quél., *Psilocybe cubensis*, and *P. cyanescens* represented different metabolic capabilities, and isolates of these fungi were selected for this study. The influence of phosphate was studied by growing the fungi in nutrient solutions differing essentially in phosphate content and by noting the distribution pattern of nitrogen between soluble and insoluble cellular components. The presence or absence of detectable quantities of key hydroxyindole derivatives was determined, and the various observations were reviewed for correlating metabolic patterns.

EXPERIMENTAL

Cultures and Nutrient Media—The isolates of *P. campanulatus* and *P. cyanescens* were prepared in 1961 from carpophores collected in western Washington. The culture of *P. cubensis* was obtained from the U.S.D.A Northern Regional Research Laboratories, Peoria, Ill., under the designation NRRL A-9109.

Catalfomo and Tyler (11) reported a medium which permitted psilocybin production when *P. cubensis* was grown in submerged cultures. Preliminary experiments showed that *P. campanulatus* and *P. cyanescens* also developed abundant growth in this medium. Thus, it was designated Medium A (Table I) and selected as a basis for modifications to accommodate the objectives of the proposed study. Determination of phosphate in the yeast extract used in test Media B, C, and D showed approximately 28.6 mg. of phosphate ion per g., and since it was considered desirable to test a

Table I—Composition of Media

Ingredient	g./l. A	B	C	D	E
Glucose	10	10	10	10	10
Ammonium succinate	1	1	1	1	1
Thiamine HCl	0.003	0.003	0.003	0.003	0.003
Difco Yeast Extract	0.5	1	1	1	0.0
KH_2PO_4	0.1	0.250	0.075	0.0	0.005
K_2SO_4	0.0	0.322	0.432	0.482	0.478
Trace minerals[a]					

Add distilled water to make 1 l. adjust, pH to 5.5 with HCl, and autoclave at 15 p.s.i. for 15 min.

[a] All media contained the following trace minerals/l.: $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.05 mg.; $ZnSO_4\cdot 7H_2O$, 0.3 mg.; $MnCl_2\cdot 4H_2O$, 0.35 mg.; $FeSO_4\cdot 7H_2O$, 2.5 mg.; $CuSO_4\cdot 5H_2O$, 0.5 mg.; $MgSO_4\cdot 7H_2O$, 500 mg.

medium with very little available phosphate, this component was omitted from Medium E. Since potassium deficiency in some higher plants is associated with a reduced incorporation of amino acids into proteins (16), various amounts of K_2SO_4 were added to the test media to provide a uniform potassium ion concentration.

Growth of Mycelia—Uniform inoculum for each fungus was considered critical. Since the experimental design projected the use of four media and the harvesting of triplicate samples after each of four incubation periods, it was necessary to have sufficient inoculum for 48 cultures. Starter cultures grown in Medium A provided the uniform inoculum. Mycelial mats were cut from three agar-slant stock cultures of each fungus and blended in a Waring micro-blender with 30 ml. of medium. Mycelia of the different fungi varied in friability and required different blending periods to obtain comparable comminution; preliminary observations resulted in the selection of 10-, 15-, and 20-sec. blending periods for *P. campanulatus*, *P. cubensis*, and *P. cyanescens*, respectively. Two-milliliter aliquots of the resulting mycelial suspension were transferred aseptically to six 500-ml. conical flasks each containing 100 ml. of medium. The culture flasks were agitated at 200 r.p.m. on a rotary Gump shaker at 25 ± 2°; after growing for 7 days, the mycelia were separated from the nutrient broths by filtering through a pad of four layers of sterile gauze with the aid of suction. The combined mycelial pellets from the six starter cultures were washed thoroughly with sterile distilled water, transferred to a Waring micro-blender, suspended in 80 ml. of sterile distilled water, and blended for the same period of time utilized for the mycelium from the agar-slant cultures. A 2-ml. volume of this suspension was pipeted aseptically to each 500-ml. conical test flask containing 100 ml. of the appropriate medium. The cultures were placed on the rotary shaker and allowed to grow at 25 ± 2° until harvested.

Approximately 9 days had been reported to permit maximum growth of *P. cubensis* (11). Incubation periods of 5, 7, 9, and 11 days were arbitrarily selected for this study. The mycelial pellets were separated by suction filtration using Whatman No. 1 filter paper. Four milliliters of nutrient broth from each culture was placed in a 4-dr. vial and retained for phosphate determination and thin-layer chromatographic evaluation. The mycelial pellets were washed thoroughly with distilled water and placed on Petri dishes left in a forced draft oven overnight at 47°. The dried material was weighed to provide a proximate indication of growth, ground to a fine powder in a mortar and pestle, and subjected to analyses for phosphate content, nitrogen distribution, and key-metabolite accumulation.

Phosphate Determination—The procedure of Weil-Malherbe and Green (17) was employed with minor modification to determine the total free and organically bound phosphate in media, culture filtrates, and dried mycelia. Accurately measured volumes (varying from 0.1 to 4.0 ml., as needed) of the media and nutrient broths were transferred to 2 × 17-cm. test tubes. The tubes were placed in a boiling water bath and an air stream directed on the solutions until the samples were evaporated to dryness. Each dried residue was digested by adding 1.2 ml. of 60% $HClO_4$ and one drop of fuming HNO_3 and heating over a microburner until the solution became clear and colorless (usually 6 to 10 min.). After cooling, 4 ml. of distilled water was added, and the solution was transferred to a 12-ml. glass-stoppered centrifuge tube. Six milliliters of isobutyl alcohol and 1 ml. of aqueous 5% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution were added to the centrifuge tube, and the mixture was shaken vigorously for 15 sec. After centrifugation, the clear isobutyl alcohol layer was transferred with a pipet to another 12-ml. centrifuge tube, approximately 2 g. of anhydrous Na_2SO_4 was added, and the mixture was agitated for 45 sec. followed by centrifugation. A 5-ml. aliquot of the isobutyl alcohol fraction was placed in a 1.6 × 15-cm. test tube with subsequent addition of 5 ml. of an acidic ethanol solution (prepared by adding 10 ml. of concentrated H_2SO_4 to 490 ml. of absolute ethanol) and 0.2 ml. of a freshly prepared, dilute solution of $SnCl_2\cdot 2H_2O$.[1] After thorough mixing, the solution was centrifuged, and the color intensity of the blue complex was measured in a Bausch & Lomb Spectronic 20 colorimeter. The maximum absorption of the blue complex was determined to be 639 mμ with a Beckman DB recording spectrophotometer, and this wavelength was employed for the colorimetric measurements. The quantity of phosphate in the sample was calculated after comparing the observed absorbance with a standard curve. The standard curve was prepared by subjecting known quantities of KH_2PO_4 to the digestion treatment and other standard manipulations.

Quantitation of phosphate in mycelia was accomplished by digesting an accurately weighed sample of the dried mycelial pellets (usually 5 to 10 mg.) with $HClO_4$ and fuming HNO_3 as described for the broth residue and processing the resultant solution in the prescribed manner. The relatively high concentrations of phosphate in the mycelia necessitated dilution of the final solution with isobutyl alcohol to permit colorimetric evaluation; tests with solutions containing known quantities of phosphate revealed that the accuracy of the assay was unaffected by the use of this alcohol as a diluent.

Nitrogen Determination—A method distinguishing free and bound amino acids or nitrogen was necessary to obtain an indication of the amino acids in fungal cells and their incorporation into proteins. Trichloroacetic acid is commonly used as a protein-precipitating agent (18), and treatment of microbial material with 10% trichloroacetic acid has been effective in extracting free amino acids while precipitating the cellular proteins (19). Separation of the cellular nitrogenous constituents into trichloroacetic acid-soluble and -insoluble fractions does not provide an absolute measure of free amino acids and proteins. Such constituents as purines and pyrimidines would be included in the trichloroacetic acid-soluble fraction, and the glucosamine content of the cell wall would contribute to the trichloroacetic acid-insoluble component. However, determination of trichloroacetic acid-soluble and -insoluble nitrogen was selected for this study since

[1] The dilute solution was prepared daily by diluting 0.5 ml. of a concentrated stock solution with sufficient 1.0 *N* H_2SO_4 to make 20 ml. Ten grams of $SnCl_2\cdot 2H_2O$ was dissolved in 25 ml. of concentrated HCl to give the stock solution.

it offered a convenient means for obtaining a qualified indication of the amino acid distribution.

Weighed samples (50–100 mg.) of the mycelia were extracted with 5-ml. volumes of 10% trichloroacetic acid in 12-ml. glass-stoppered centrifuge tubes placed horizontally in a rack clamped to the rotary shaker. After shaking for 1 hr., the tubes were centrifuged, and the clear supernatant solutions were decanted into 30-ml. Kjeldahl flasks. The marcs were reextracted with second 5-ml. portions of 10% trichloroacetic acid for 1 hr. These second supernatants were added to the initial extracts, and the volumes of liquid were evaporated to approximately 0.5 ml. each by heating the flasks in a boiling water bath while directing an air stream on the solutions. The concentrated extracts were subjected to standard Kjeldahl digestions, distillations, and titrations for nitrogen determination.

Marcs remaining after removal of the trichloroacetic acid-soluble materials were digested in 30-ml. Kjeldahl flasks and processed to quantitate the trichloroacetic acid-insoluble nitrogen.

Detection of Indole Metabolites—The thin-layer chromatographic system A of Leung *et al.* (12) was suitable for rapid, sensitive evaluation of the hydroxyindole metabolites of *Panaeolus* and *Psilocybe* species. Weighed portions (usually 50 mg.) of the dried, powdered mycelia were extracted by shaking with 5 ml. of methanol in 12-ml. glass-stoppered centrifuge tubes for 1 hr. The mixtures were centrifuged, and the clear supernatant solutions were removed with pipets. The marcs were extracted with second 5-ml. volumes of methanol. Preliminary experiments showed that no detectable quantities of tryptamines were present in the supernatant from a third extraction of mycelium known originally to contain a relatively high level of these indoles. The two extracts were combined in a 50-ml. round-bottom flask and evaporated to dryness under reduced pressure at 40° in a flash evaporator. The soluble metabolites in the residue from each sample were dissolved in 0.5 ml. of methanol, and 10-μl. volumes of the extracts were chromatographed to determine if psilocybin, psilocin, serotonin, or 5-hydroxytryptophan was detectable.

Various volumes (0.1 to 10 μl.) of those extracts containing detectable quantities of one or more of the key metabolites were chromatographed to permit comparison of spot size with that of a known quantity of pure reference material and to permit determination of the volume of extract containing the minimum detectable quantity of the constituent. This information was utilized to estimate the approximate concentration of the metabolite in the mycelium. Table II shows the relative migrations of the four key hydroxyindole derivatives and lists the minimum detectable quantities of the pure compounds under the experimental conditions.

Table II—Thin-Layer Chromatographic Characteristics of Key Hydroxyindole Derivatives[a]

Compound	*Rf*	Minimum Detectable Quantity, mcg.
Psilocybin	0.19	0.05
Psilocin	0.79	0.05
Serotonin	0.63	0.05
5-Hydroxytryptophan	0.57	0.025

[a] The chromatographic adsorbant was a mixture of Silica Gel G:Kieselguhr G (2:1), and the solvent system was *n*-propanol:5% NH_4OH (5:2). The chromatograms were sprayed with 2% *p*-dimethylaminobenzaldehyde in acidic ethanol to produce visible chromophores.

Catalfomo *et al.* (11) reported the absence of hydroxyindole derivatives in the nutrient broth from cultures of *P. cubensis* which had produced such metabolites. Presumably, these constituents are retained endocellularly. However, the broth from one flask in each triplicate set was examined for the possible excretion of this type of metabolite. Two-milliliter samples of the nutrient solutions were evaporated to dryness under reduced pressure, methanolic extracts of the residues were prepared, and the extracts were evaluated chromatographically.

RESULTS AND DISCUSSION

A common inoculum was used for the test flasks of each species in the study to provide maximum uniformity of the fungi prior to their growth in the various media. The test flasks were subjected to constant environmental conditions for the designated

Table III—Average Growth and Distribution of Nitrogen and Phosphate in Cultures of *P. campanulatus*

Culture Series[a]	Mycelial Dry Wt., mg.	Mycelial TCA-sol. N[b] mg./flask	%	Mycelial TCA-insol. N mg./flask	%	Ratio, TCA-sol. N/TCA-insol. N	Added Phosphate,[c] mcg./ml.	Phosphate: Mycelium mcg./mg.	Phosphate: Medium mcg./ml.
5B	538 ± 13	3.03 ± 0.10	0.56	15.2 ± 0.1	2.82	0.20	178	16.0 ± 0.3	4.5 ± 0.7
5C	425 ± 11	2.94 ± 0.16	0.69	15.6 ± 0.1	3.66	0.19	65.0	8.6 ± 0.2	0.60 ± 0.07
5D	175 ± 4	2.80 ± 0.04	1.60	6.2 ± 0.2	3.56	0.45	14.3	6.7 ± 0.2	0.64 ± 0.14
5E	93 ± 3	1.23 ± 0.07	1.31	2.9 ± 0.2	3.14	0.41	3.37	5.4 ± 0.1	0.52 ± 0.11
7B	356 ± 1	3.86 ± 0.13	1.08	10.2 ± 0.3	2.88	0.37	178	23.4 ± 1.0	4.1 ± 0.4
7C	403 ± 5	4.18 ± 0.40	1.04	13.8 ± 0.1	3.44	0.30	65.0	8.2 ± 0.4	0.78 ± 0.05
7D	187 ± 2	2.47 ± 0.05	1.32	6.3 ± 0.1	3.42	0.39	14.3	6.3 ± 0.1	0.38 ± 0.04
7E	98 ± 10	1.09 ± 0.10	1.11	2.6 ± 0.3	2.70	0.41	3.37	5.0 ± 0.2	0.30 ± 0.07
9B	315 ± 5	4.44 ± 0.19	1.41	7.2 ± 0.1	2.28	0.62	178	24.5 ± 0.5	15.2 ± 0.5
9C	330 ± 6	4.14 ± 0.01	1.25	9.2 ± 0.2	2.78	0.45	65.0	10.6 ± 0.4	1.2 ± 0.1
9D	228 ± 9	2.45 ± 0.07	1.07	7.6 ± 0.3	3.31	0.33	14.3	5.3 ± 0.0	0.50 ± 0.04
9E	105 ± 1	1.19 ± 0.04	1.13	2.8 ± 0.1	2.62	0.43	3.37	4.6 ± 0.2	0.35 ± 0.00
11B	313 ± 2	4.17 ± 0.07	1.34	6.9 ± 0.1	2.24	0.59	178	23.5 ± 0.8	21.6 ± 0.4
11C	331 ± 5	3.79 ± 0.07	1.14	8.0 ± 0.3	2.43	0.47	65.0	10.7 ± 0.6	1.3 ± 0.0
11D	261 ± 9	2.62 ± 0.07	1.01	8.3 ± 0.4	3.17	0.32	14.3	4.6 ± 0.1	0.58 ± 0.12
11E	124 ± 10	1.30 ± 0.14	1.00	3.4 ± 0.3	2.72	0.38	3.37	4.2 ± 0.0	0.38 ± 0.04

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] Trichloroacetic acid. [c] Determined by assay of aliquots of uninoculated media.

TABLE IV—AVERAGE GROWTH AND DISTRIBUTION OF NITROGEN AND PHOSPHATE IN CULTURES OF *P. cubensis*

Culture Series[a]	Mycelial Dry Wt., mg.	Mycelial TCA-sol. N[b] mg./flask	%	Mycelial TCA-insol. N mg./flask	%	Ratio, TCA-sol. N/TCA-insol. N	Added Phosphate,[c] mcg./ml.	Phosphate Mycelium mcg./mg.	Phosphate Medium mcg./ml.
5B	397 ± 8	6.62 ± 1.05	1.68	9.4 ± 0.3	2.37	0.72	190	12.9 ± 0.9	38.0 ± 0.0
5C	432 ± 37	3.21 ± 0.34	0.74	15.3 ± 1.4	3.54	0.21	78.4	9.1 ± 0.4	2.8 ± 0.0
5D	226 ± 2	3.64 ± 0.30	1.61	7.6 ± 1.6	3.37	0.50	16.0	7.3 ± 0.8	1.5 ± 0.3
5E	86 ± 8	0.85 ± 0.05	1.00	3.4 ± 0.4	3.94	0.25	3.50	7.7 ± 0.3	0.48 ± 0.04
7B	313 ± 9	5.38 ± 0.52	1.72	9.6 ± 0.6	3.07	0.57	190	18.0 ± 0.7	51.0 ± 1.3
7C	367 ± 18	4.34 ± 0.19	1.29	15.2 ± 0.7	4.13	0.31	78.4	9.5 ± 0.6	4.9 ± 0.0
7D	270 ± 13	4.06 ± 0.21	1.50	10.8 ± 0.4	3.97	0.38	16.0	6.6 ± 0.1	0.56 ± 0.00
7E	100 ± 14	0.91 ± 0.19	0.90	4.2 ± 0.4	4.24	0.22	3.50	6.9 ± 0.1	0.44 ± 0.01
9B	263 ± 6	3.61 ± 0.74	1.37	9.9 ± 0.5	3.77	0.36	190	18.0 ± 2.0	61.0 ± 1.40
9C	347 ± 5	5.82 ± 0.44	1.68	13.8 ± 0.9	3.97	0.43	78.4	11.3 ± 0.4	1.1 ± 0.1
9D	324 ± 18	4.52 ± 0.17	1.40	11.6 ± 0.8	3.55	0.39	16.0	5.7 ± 0.1	0.63 ± 0.04
9E	98 ± 2	0.77 ± 0.06	0.78	4.3 ± 0.1	4.40	0.18	3.50	7.1 ± 0.2	0.48 ± 0.04
11B	257 ± 4	4.07 ± 0.23	1.58	9.0 ± 0.2	3.52	0.45	190	15.0 ± 2.0	70.0 ± 1.3
11C	324 ± 19	5.69 ± 0.18	1.77	11.8 ± 0.2	3.64	0.49	78.4	14.0 ± 2.0	2.8 ± 0.0
11D	325 ± 7	4.92 ± 0.26	1.51	12.5 ± 0.4	3.85	0.39	16.0	5.5 ± 0.3	0.83 ± 0.04
11E	113 ± 10	0.83 ± 0.09	0.73	4.9 ± 0.5	4.30	0.17	3.50	6.0 ± 0.5	0.35 ± 0.00

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] Trichloroacetic acid. [c] Determined by assay of aliquots of uninoculated media.

incubation periods, and triplicate sets of flasks were analyzed for each variable to minimize the possibility of using extreme values in the formulation of indicative growth and metabolic patterns.

Analytical data for the distribution of nitrogen and phosphate in the cultures are given in Tables III, IV, and V. Growth of all three fungi was related grossly to the phosphate content of the media. The reduced growth rates in Medium D suggested the possibility that phosphate-deficient metabolic patterns might be detectable in cultures grown in this nutrient solution. The very poor growth in Medium E was probably a combined result of limited availability of phosphate and of unavailability of some factor(s) from the yeast extract which was omitted from this medium.

The data showed that phosphate was readily removed from the media by all three species. *P. campanulatus* was particularly efficient in concentrating phosphate in the mycelium. *P. cyanescens* demonstrated the least tendency to concentrate phosphate in the fungal cells, and when this fungus was grown in Medium B, the residual phosphate in the culture broth clearly indicated that this nutrient was available in excess of the requirements of the fungus and in excess of the strain's capacity to store phosphate. *P. campanulatus* and *P. cubensis* grew equally well in Media B and C, indicating the additional phosphate in Medium B was not contributing to the growth of the fungi.

Distribution of phosphate in the various culture broths confirmed the anticipated exocellular release of phosphate from the older cells. Detection of this phenomenon should be easiest in cultures which develop rapidly in phosphate-rich media and which are harvested after longer incubation periods. *P. cubensis* grown in Medium B provided the most pronounced example observed during this investigation.

Evaluation of data on mycelial phosphate, especially that obtained with mycelia from phosphate-poor cultures, requires special attention. Calculation of the total phosphate per flask will reveal more phosphate than was present in the nutrient medium. This situation reflects the endogenous phosphate which was incorporated into the culture flasks with the inoculum. The quantity of phosphate introduced with the inoculum was insignificant for phosphate-rich cultures, but it represented an increasingly larger proportion of the total in flasks with media containing lesser amounts of KH_2PO_4.

TABLE V—AVERAGE GROWTH AND DISTRIBUTION OF NITROGEN AND PHOSPHATE IN CULTURES OF *P. cyanescens*

Culture Series[a]	Mycelial Dry Wt., mg.	Mycelial TCA-sol. N[b] mg./flask	%	Mycelial TCA-insol. N mg./flask	%	Ratio, TCA-sol. N/TCA-insol. N	Added Phosphate,[c] mcg./ml.	Phosphate Mycelium mcg./mg.	Phosphate Medium mcg./ml.
5B	443 ± 5	3.01 ± 0.09	0.68	11.9 ± 0.2	2.68	0.25	164	8.7 ± 0.2	66.0 ± 3.0
5C	452 ± 16	2.78 ± 0.14	0.62	11.7 ± 0.2	2.59	0.24	65.6	7.1 ± 0.4	3.3 ± 0.0
5D	241 ± 6	1.78 ± 0.09	0.74	7.1 ± 0.0	2.96	0.25	15.2	4.9 ± 0.0	0.74 ± 0.09
5E	100 ± 1	0.52 ± 0.06	0.52	3.0 ± 0.0	3.00	0.17	2.90	4.7 ± 0.1	0.30 ± 0.04
7B	413 ± 5	2.23 ± 0.07	0.54	11.6 ± 0.3	2.82	0.19	164	8.9 ± 0.4	69.0 ± 1.3
7C	426 ± 5	2.18 ± 0.15	0.51	12.5 ± 0.2	2.92	0.18	65.6	7.3 ± 0.2	5.5 ± 0.2
7D	311 ± 7	2.00 ± 0.22	0.64	8.5 ± 0.2	2.74	0.23	15.2	4.0 ± 0.1	0.67 ± 0.09
7E	136 ± 2	0.52 ± 0.15	0.55	3.7 ± 0.1	2.71	0.14	2.90	4.0 ± 0.0	0.49 ± 0.18
9B	365 ± 3	1.84 ± 0.17	0.50	12.8 ± 0.2	3.50	0.14	164	9.3 ± 0.4	68.0 ± 2.3
9C	386 ± 7	1.88 ± 0.25	0.49	12.9 ± 0.9	3.36	0.15	65.6	8.5 ± 0.1	5.3 ± 0.3
9D	300 ± 7	1.74 ± 0.06	0.58	8.3 ± 0.5	2.75	0.21	15.2	4.1 ± 0.1	0.72 ± 0.09
9E	135 ± 8	0.73 ± 0.04	0.55	4.3 ± 0.1	3.19	0.17	2.90	4.0 ± 0.2	0.39 ± 0.04
11B	362 ± 4	2.02 ± 0.10	0.56	11.7 ± 0.2	3.23	0.17	164	9.6 ± 0.2	71.0 ± 1.7
11C	362 ± 14	1.85 ± 0.05	0.51	11.4 ± 0.6	3.14	0.16	65.6	8.8 ± 0.2	5.1 ± 0.0
11D	260 ± 10	1.62 ± 0.15	0.62	7.6 ± 0.1	2.91	0.21	15.2	4.3 ± 0.1	0.81 ± 0.02
11E	148 ± 11	0.73 ± 0.08	0.49	4.2 ± 0.3	2.85	0.17	2.90	3.8 ± 0.1	0.49 ± 0.09

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] Trichloroacetic acid. [c] Determined by assay of aliquots of uninoculated media.

The quantities of trichloroacetic acid-soluble and -insoluble nitrogen in the mycelia were considered indicative of the free amino acid pool and the protein content, respectively. Various carbohydrate metabolites normally constitute a major portion of the mycelial dry weight, and data on the nitrogenous fractions were evaluated on the basis of nitrogen content per unit weight of mycelium and per flask to reveal changes in the distribution of nitrogenous constituents which were independent of changes in the carbohydrate metabolites. Ratios of the trichloroacetic acid-soluble and -insoluble nitrogen were particularly useful for easy detection of a relative redistribution of nitrogenous metabolites between the two fractions.

Comparison of the maximum mycelial dry weights and the incorporation of nitrogen into trichloroacetic acid-insoluble metabolites suggested that *P. cubensis* reached maximum growth prior to the maximum formation of proteins. A similar, but less pronounced, tendency appeared to exist for *P. cyanescens*. A contrasting pattern in which maximum growth and protein formation were more nearly concurrent was noted for *P. campanulatus*. The majority of the mycelial nitrogen occurred in the bound form in the three species under all of the experimental conditions. The highest concentrations of soluble nitrogen and the highest ratios of soluble to insoluble nitrogen were observed with cultures of *P. cubensis* and *P. campanulatus*. *P. cyanescens* appeared to have a limited tendency for accumulation of soluble nitrogenous metabolites.

The most striking increases in soluble nitrogen during the progressive incubation periods were observed in mycelia of *P. campanulatus* grown in Media B and C and of *P. cubensis* grown in Medium C. The increase in soluble nitrogen in the mycelium of *P. cubensis* was accompanied by a corresponding decrease in the total insoluble nitrogen in the fungal tissue, and it appeared that the net increase in free amino acid nitrogen was probably a result of protein catabolism. However, only a modest net increase in soluble nitrogen was associated with a marked decrease in the total insoluble nitrogen in the mycelia of *P. campanulatus* growth in Media B and C; the decreased total mycelial nitrogen indicated exocellular excretion of nitrogen metabolites by this species during the longer incubation periods.

Chromatographic examination of the mycelial samples for psilocybin, psilocin, 5-hydroxytryptophan, or serotonin revealed detectable quantities of psilocybin and psilocin in *P. cubensis* grown in certain media for appropriate incubation periods (Table VI). None of the key metabolites was detected in mycelia of *P. campanulatus* or *P. cyanescens* or in any of the nutrient broths. When the indoles were not detected following chromatographic evaluation of 10 μl. of an extract prepared from 50 mg. of dried mycelium, the levels of accumulation of psilocybin, psilocin, and serotonin were less than 0.005%, and the concentration of 5-hydroxytryptophan was less than 0.0025%. Other components in the mycelial extracts prevented good resolution when more than 10-μl. quantities were applied to the chromatograms. Minimum concentrations of the compounds which could have been detected in the media were 1.25 mcg./ml. of psilocybin, psilocin, and serotonin and 0.63 mcg./ml. of 5-hydroxytryptophan.

TABLE VI—OCCURRENCE AND ESTIMATED ACCUMULATION OF PSILOCYBIN AND PSILOCIN IN MYCELIUM OF *P. cubensis*

Culture Series[a]	Psilocybin, mcg./mg.	Psilocin, mcg./mg.
5B	—[b]	—
5C	0.1	—
5D	0.1	—
5E	—	—
7B	0.5	—
7C	0.5	0.1
7D	0.5	—
7E	—	—
9B	2.0	0.2
9C	0.5	—
9D	0.05	—
9E	—	—
11B	2.0	—
11C	1.0	—
11D	0.2	—
11E	—	—

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] None detectable.

Accumulation of psilocybin in the mycelium of *P. cubensis* appeared to have some relationship to phosphate nutrition and general nitrogen metabolism. Formation of this metabolite occurred predominantly after maximum growth, and the results with Medium B suggested that excess phosphate in the mycelium may delay or suppress secondary tryptamine metabolism in this fungus. The formation of secondary nitrogenous constituents after growth-associated somatic protein synthesis has ceased to remove large quantities of amino acids from the soluble nitrogen pool is consistent with theoretical considerations and corresponds to the pattern recently reported with alkaloid formation in *Claviceps* strain SD58 (20). Catalfomo *et al.* (11) utilized a nutrient solution similar to Medium C in studies of *P. cubensis* and reported maximum psilocybin production after a 7-day incubation period and maximum growth after 9 days; the long incubation period required to reach maximum growth suggests that relatively small quantities of inoculum were utilized in the investigation, and a mixture of cells representing widely different stages of metabolic activity may account for the apparent maximum psilocybin content during the growth phase of the organism. The quantity of inoculum employed in this investigation was sufficient to permit rapid growth of the fungus when other conditions were favorable, and the observed tendency for psilocybin formation during the stationary phase of the culture appears valid.

CONCLUSIONS

The studies revealed that saprophytic mycelium of *P. cubensis* was characterized by a relatively high soluble nitrogen component and by the capacity to accumulate secondary tryptamine metabolites under selected conditions. Mycelium of the closely related *P. cyanescens* lacked the capacity to accumulate appreciable quantities of soluble nitrogenous compounds or detectable quantities of key tryptamines. Experimental design precluded interpretation of cause and effect, so there is no direct evidence that a

limited free amino acid pool in *P. cyanescens* is responsible for the failure of the fungus to accumulate psilocybin in saprophytic cultures. However, the results do suggest that the isolate of *P. cyanescens* is less responsive to phosphate nutrients and has narrower metabolic tolerance than *P. cubensis* NRRL A-9109.

P. campanulatus appeared to be the most metabolically responsive of the species tested. It grew well under the saprophytic conditions and appeared to have an adequate soluble nitrogen pool. These observations suggest the improbability of a limited supply of common amino acids as the basic factor behind the lack of serotonin production in saprophytic cultures. Some key factor controlling the formation or function of tryptophan hydroxylase is probably responsible for the different metabolic capabilities of carpophores and vegetative mycelium of this basidiomycete.

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Keyphrases

Basidiomycete cultures—phosphate effect
Phosphate effect—trichloroacetic acid-soluble, -insoluble nitrogen metabolites
Fungal free amino acid pool—hydroxytryptamine derivatives accumulation
Colorimetric analysis—spectrophotometer
TLC—separation, identity

Baeocystin and Norbaeocystin: New Analogs of Psilocybin from *Psilocybe baeocystis*

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Two new 4-phosphoryloxytryptamine derivatives have been isolated from *Psilocybe baeocystis* grown in submerged cultures. Using ultraviolet, infrared, and mass spectral analyses the structures have been determined. Both are analogs of the psychotomimetic, psilocybin, and have been named baeocystin (monomethyl analog) and norbaeocystin (demethyl analog).

PSILOCYBIN was first isolated from a Mexican psychotomimetic mushroom, *Psilocybe mexicana* Heim, by Hofmann *et al.* (1). While the occurrence of this compound and its dephosphorylated derivative, psilocin, has been established in several species of mushrooms, analogs have not yet been described.

The formation of psilocybin in submerged cultures of *Psilocybe baeocystis* Singer and Smith has been reported previously (2). In a communication (3) the authors also have reported the isolation of baeocystin, a monomethyl analog of psilocybin, from this same fungus. The present report deals with details of the procedure for the isolation and characterization

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