The Relationship of Carbon and Nitrogen Nutrition of *Psilocybe baeocystis* to the Production of Psilocybin and its Analogs

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Investigations of the culture of psychotomimetic fungi have been meager. Surface culture of a limited number of species has been described by Singer (11) and Heim *et al.* (5–7). Submerged culture of psychotropic fungi was first attempted by Catalfomo and Tyler (2). They established conditions favoring the formation of mycelial pellets of *Psilocybe cubensis* which contained psilocybin. However, *P. cyanescens* and *P. pelliculosa* failed to produce detectable amounts of psilocybin under the same conditions.

There are no reports of investigations of the influence of various carbon and nitrogen sources on the growth of psychotomimetic fungi and on the production of psychedelic compounds. An objective of the present study was to investigate the effects of certain carbon and nitrogen sources on the growth of *P. baeocystis* Singer and Smith, in submerged cultures, and on the production of psilocybin and its analogs, baeocystin and norbaeocystin (8).

MATERIAL AND METHODS

Organism.—The culture of *P. baeocystis* has been described in a previous report (9). Stock cultures of this species were maintained on potato dextrose agar at 4°.

Carbon and nitrogen sources.—The carbon sources selected for these investigations were sucrose, lactose, maltose, trehalose, fructose, glucose and galactose. The nitrogen sources used were glycine, ammonia, nitrate and urea.

Composition of media.—A modification of Catalfomo and Tyler's medium (2) was used as the control medium for both carbon and nitrogen nutritional studies, SM–1 (9).

For carbon nutrition studies, glucose in SM–1 was replaced by the test carbon source. The concentration in each case was 0.3% carbon.

For nitrogen nutrition studies, glycine and ammonium succinate in SM–1 were replaced by an equivalent amount (0.19% nitrogen) of the test nitrogen source.

In experiments using glycine, ammonium succinate was omitted from SM–1 and the glycine concentration was increased to supply the required nitrogen concentration. The pH was adjusted to 5.5 with 0.5N HCl.

For experiments involving ammonia as the sole nitrogen source, glycine was eliminated from SM–1 and 7.9230 g/liter of succinic acid and 8.9 ml/liter of ammonia were used. The pH was adjusted to 5.5 with 10% KOH.

In experiments using nitrate, glycine and ammonium succinate were replaced by 13.56 g/liter of KNO_3. The pH was adjusted to 5.5 with 10% KOH.

In the preparation of the medium for experiments using urea, glycine and ammonium succinate were omitted from SM–1. The pH of the medium was adjusted to 5.5 with 10% KOH, and the medium was diluted to 900 ml with distilled water. Ninety ml of this medium was transferred to each of ten 500-ml culture flasks and sterilized. A 4.03% aqueous solution of urea was prepared and sterilized by filtering through a sterile Morton bacteria filter apparatus. Ten ml of this solution was pipetted aseptically into each of the flasks.

Preparation of submerged cultures.—The general procedure for preparing the inocula has been described in a previous publication (9). In the present investigations, tissue suspensions of 50% transmittance were used as inocula. Using 10-ml sterile serological pipettes, 2 ml of the suspension was transferred aseptically to each of four 500-ml culture flasks containing 100 ml of a particular medium. The remaining 2 ml of inoculum in the pipette was discarded. The flasks were incubated in a rotary shaker as previously described (9).

Dry weight determination.—Tissue pellets were separated from the culture medium by filtering through muslin, washed on the filter with several ml of distilled water and transferred to a weighing bottle. They were freeze-dried for 36 to 48 hr. The tissues in the respective weighing bottles were stored in a desiccator over silica gel until used. Weighings were made on a Metler semi-micro balance. After weighing, tissue pellets were ground to a fine powder and stored in the desiccator.

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Preparation of tissue extracts.—In order to minimize inaccuracies due to excessive handling of tissue material, a simple extraction method (1) requiring no filtration or transferring of the extract was followed. The powdered tissue was weighed on the Metler balance and transferred to a 4-dram screwcap vial. Using a volumetric pipette, sufficient methanol was added to yield a concentration of 2 or 2.5 mg of tissue/ml of solvent. The vial was tightly stoppered and agitated, at room temperature, on the rotary shaker for 24 to 48 hrs. The vials were centrifuged and stored in a freezer without decanting. They were removed from the freezer approximately 30 min. prior to use.

Quantitative analyses of psilocybin and its analogs.—A quantitative thin-layer chromatographic (TLC) procedure was developed. Psilocybin was used as the reference standard throughout the assay procedures on the assumption that analogs of psilocybin possessing the same substituted indole nucleus give identical color reactions with Ehrlich's reagent (5% *p*-dimethylaminobenzaldehyde in concentrated HCl).

A series of amounts of reference psilocybin ranging from 0.1 to 0.5 γ were spotted on a plate of silica gel G-kieselguhr G (2:1), and increasing volumes of a tissue extract were spotted on the same plate. The plate was developed with solvent system A (9). After spraying with Ehrlich's reagent, the plate was allowed to stand at room temperature for 20 to 30 min and the color intensities were compared visually. The concentrations of reference and sample compounds which gave the weakest color were noted and matched, and the fungal content of psilocybin and its analogs was calculated.

RESULTS

Experiments were carried out in triplicate. The cultures were harvested after 2, 3 and 4 weeks of incubation, and the pH of the culture media was determined at the time of harvest.

Carbon nutrition.—The data obtained are summarized in table 1. The psilocybin analogs reported are a mixture of baeocystin and norbaeocystin, with baeocystin in predominate concentration.

TABLE 1. *Results of carbon nutrition studies.*

Carbon Source	pH	Growth Weight (mg)	Compounds (%)	
			Psilocybin	Psilocybin Analogs
2 Weeks				
Glucose	5.1±0.2	46.9±11.8	1.45±0.17	0.12±0.01
Fructose	5.2±0.0	39.9± 2.6	0.18±0.03	<0.04
Galactose	5.3±0.1	17.5± 0.6	1.87±0.44	0.12±0.03
Maltose	5.0±0.1	65.1±10.0	0.83±0.24	0.10±0.01
Trehalose	5.4±0.0	32.8± 4.9	1.31±0.08	0.10±0.02
Sucrose	- - -[a]	no growth	- - -	- - -
Lactose	- - -	no growth	- - -	- - -
3 Weeks				
Glucose	4.4±0.1	141.9±15.5	0.43±0.05	0.09±0.01
Fructose	5.1±0.0	88.3± 5.1	0.25±0.02	<0.03
Galactose	5.3±0.0	22.9± 1.0	0.70±0.14	<0.03
Maltose	4.4±0.1	161.1±13.5	0.25±0.09	<0.03
Trehalose	4.8±0.0	110.5± 4.3	0.89±0.08	0.04±0.01
Sucrose	5.7±0.0	12.9± 0.8	0.55±0.10	<0.03
Lactose	- - -	no growth	- - -	- - -
4 Weeks				
Glucose	4.3±0.1	168.3±19.9	0.08±0.02	<0.03
Fructose	5.1±0.1	112.4± 2.8	0.16±0.10	<0.03
Galactose	- - -	- - -	- - -	- - -
Maltose	4.6±0.3	222.5± 9.6	0.08±0.02	<0.03
Trehalose	4.6±0.0	160.8± 8.1	0.43±0.05	<0.03
Sucrose	- - -	meager growth	- - -	- - -
Lactose	- - -	no growth	- - -	- - -

[a] - - - =not determined.

Of the seven sources tested, lactose did not support any growth of the fungus, while sucrose and glactose only supported meager growth. However, the percentage content of psilocybin in tissues grown in the medium with galactose appeared to be the highest. The total amounts of psilocybin and its analogs produced are shown in table 2.

TABLE 2. *Total content*[a] *(μg) of psilocybin and psilocybin analogs in nutritional studies.*

	2 Weeks		3 Weeks		4 Weeks	
	Psilocybin	Psilocybin Analogs	Psilocybin	Psilocybin Analogs	Psilocybin	Psilocybin Analogs
Carbon Source						
Glucose	680	56	610	128	135	———
Fructose	323	———[b]	221	———	180	———
Galactose	327	21	160	———	- - -[b]	- - -
Maltose	540	65	403	———	178	———
Trehalose	430	33	984	44	692	———
Sucrose	- - -	- - -	71	———	- - -	- - -
Lactose	- - -	- - -	- - -	- - -	- - -	- - -
Nitrogen Source						
Control	1210	92	914	199	441	———
Glycine	397	———	418	———	569	———
Urea	———	———	———	———	- - -	- - -
Ammonia	- - -	- - -	- - -	- - -	13	———
Nitrate	- - -	- - -	- - -	- - -	- - -	- - -
Control minus glycine	98	———	106	———	———	———
Control minus ammonia	416	———	394	———	124	———

[a]Total content = percentage x growth weight.
[b]——— = not detected, - - - = not determined.

Fructose was inferior to glucose both in supporting growth of *P. baeocystis* and in the production of psilocybin and its analogs.

Although maltose seemed to support better growth of *P. baeocystis* than glucose, the concentrations of psilocybin and its analogs produced were lower. A comparison of the total contents of these compounds (table 2) indicates that glucose is a better source for their production.

Trehalose was comparable with glucose in supporting the growth of the fungus and the yield of psilocybin was high. However, the concentration of psilocybin analogs was low.

Experiments using the control medium from which glucose was omitted showed that *P. baeocystis* could not utilize succinate as the sole source of carbon.

Nitrogen nutrition.—The results (table 3) show that the control nitrogen source (0.17% glycine N and 0.02% ammonia N) is the best for *P. baeocystis*, for growth and the production of psilocybin and its analogs. It is interesting to note that psilocybin analogs were not produced in detectable concentrations by any of the other sources.

Addition of 50 mg/100 ml of L-tryptophan to the control medium did not seem to influence the formation of psilocybin and its analogs (table 4).

DISCUSSION

Nutritional studies.—Previous studies by the present authors (9) showed that two complex media, Sabouraud's liquid medium and malt extract broth, supported good growth of *P. baeocystis* but failed to support the formation of psilo-

TABLE 3. *Results of nitrogen nutrition studies.*

Nitrogen Source	pH	Growth Weight (mg)	Compounds (%)	
			Psilocybin	Psilocybin Analogs
2 Weeks				
Control	4.8±0.1	92.4±13.3	1.31±0.22	0.10±0.08
Glycine	4.6±0.1	36.4± 8.3	1.09±0.16	<0.03
Urea	8.6±0.0	12.5± 1.4	<0.05	<0.05
Ammonia	5.6	no growth	- - -[a]	- - -
Nitrate	4.5	no growth	- - -	- - -
Control minus glycine	5.0±0.1	70.3±16.3	0.14±0.05	<0.03
Control minus ammonia	4.6±0.0	40.9±16.7	0.85±0.17	<0.03
3 Weeks				
Control	4.5±0.1	198.5±32.0	0.46±0.06	0.10±0.02
Glycine	4.6±0.1	91.0±17.9	0.46±0.06	<0.03
Urea	8.1±0.0	7.5±0.5	<0.05	<0.05
Ammonia	5.6	no growth	- - -	- - -
Nitrate	4.4	no growth	- - -	- - -
Control minus glycine	4.3±0.3	132.8±27.2	0.08±0.04	<0.03
Control minus ammonia	4.6±0.0	75.8± 9.1	0.52±0.11	<0.03
4 Weeks				
Control	4.2±0.1	315.2± 7.9	0.14±0.02	<0.03
Glycine	4.4±0.0	189.7±20.0	0.30±0.04	<0.03
Urea	7.9	3.4± 1.1	- - -	- - -
Ammonia	5.5±0.0	21.8±13.3	0.06±0.01	<0.03
Nitrate	- - -	no growth	- - -	- - -
Control minus glycine	3.8±0.1	155.1± 9.3	<0.03	<0.03
Control minus ammonia	4.5±0.0	88.5± 7.3	0.14±0.03	<0.03

[a] - - -=not determined.

TABLE 4. *Results of tryptophan nutrition studies.*

	pH	Growth Weight (mg)	Compounds (%)	
			Psilocybin	Psilocybin Analogs
2 weeks				
Control	5.1±0.2	51.6± 3.5	0.93±0.09	0.13±0.05
Control plus tryptophan	4.8±0.1	85.4± 8.4	0.93±0.09	0.12±0.03
3 Weeks				
Control	4.3±0.2	111.5±31.6	0.63±0.26	0.13±0.09
Control plus tryptophan	4.4±0.0	122.3±13.7	0.53±0.05	0.05±0.02
4 Weeks				
Control	4.5±0.2	209.6±12.0	0.33±0.05	<0.03
Control plus tryptophan	4.3±0.1	320.5±39.4	0.37±0.05	<0.03

cybin or its analogs. Both media are rich in amino nitrogen and minerals. The reason of their failure in supporting the production of these compounds is unknown.

Results obtained from the present investigations do not show any parallelism between growth and the formation of psilocybin and its analogs (tables 1 and 3). In all cases, psilocybin accumulation was highest in 2 weeks, but decreased rapidly in 3 and 4 weeks while growth continued to increase. Hence, good growth alone cannot be considered as a favorable result. It must also be accompanied by the accumulation of these compounds. Consequently, it was found desirable to consider the total amount of psilocybin and its analogs present in a culture.

Results from carbon nutritional studies (table 2) indicate that, of the sugars tested, glucose (control) served as the best carbon source for the production of psilocybin and its analogs. Although maltose seemed to support better growth, the total yield of these compounds was lower, and while the percentage of these compounds was greater when galactose was used, the meager growth resulted in a lower total yield.

In the presence of trehalose, the total yield of psilocybin was greater than for the other sugars tested. However, the yields of psilocybin analogs were low.

Sucrose gave meager growth and little psilocybin could be detected, while lactose failed to support growth of *P. baeocystis*. These results are consistent with those obtained for related fungi. Of 12 species of *Coprinus* tested with lactose by Fries (4), none showed good growth, and of 7 species of *Tricholoma* investigated by Norkrans (11), only *T. nudum* grew well in lactose. Similar results were obtained with sucrose (4, 11).

Results from nitrogen nutritional studies (table 3) have shown that at a nitrogen concentration of 0.19%, glycine is the best nitrogen source among the four sources tested singly.

Nitrate, as would be expected for higher fungi (3), did not support any growth of *P. baeocystis*.

Urea only supported very slight growth, and no psilocybin or its analogs could be detected in tissues obtained using this source. Urea has been shown to be effectively utilized by various species of *Tricholoma* and *Coprinus* for growth (4, 11) at concentrations of 0.05% and 0.01% of nitrogen respectively. Its concentration in the present studies (0.19%) was probably too high.

Ammonia nitrogen at a concentration of 0.19% did not support growth of *P. baeocystis* up to 3 weeks, and only meager growth after 4 weeks. This may be due to inhibitory effects of ammonia nitrogen at this concentration. This phenomenon was observed by Norkrans (11). In an experiment with a *Tricholoma* species using 5, 1, and 0.2 g/liter of ammonium tartrate as the sole source of nitrogen, growth was found to be poorest in the 5-g/liter medium, while it was about the same in the media containing 1 and 0.2 g/liter. The data obtained from the present investigations suggest a similar inhibitory effect produced by a high concentration of ammonia nitrogen. Thus, when glycine was omitted from the control medium, the remaining ammonia nitrogen (0.02%) in the medium was able to support good growth of *P. baeocystis*.

A combination of glycine and ammonia nitrogen (control) was found to be the best nitrogen source for *P. baeocystis* both for growth and for the production of psilocybin and its analogs. Either source alone did not support the production of psilocybin analogs in the organism and resulted in a lower yield of psilocybin. This was not observed with *P. cubensis* (2).

No attempts have been made in these studies to determine the optimal concentrations of the carbon and nitrogen sources for growth and for the production of psilocybin and its analogs. Similarly, no attempts were made to determine the peak growth of the fungus or the peak-production of psychotomimetic compounds. Hence, failure of the fungus to utilize a certain source may simply be due, among other factors, to the unfavorable or toxic concentrations of the source employed.

Quantitative TLC technique.—This technique involves visual comparisons of the minimum detectable color intensities of psilocybin and its analogs on developed and sprayed chromatograms. A similar technique on paper has been reported for the determination of psilocybin produced by *P. cubensis* in submerged culture (2). One major factor which affects the results when using the TLC method is the non-uniformity of the plates. This defect was overcome in the present investigations by the development of an improved plate-preparation procedure. In this procedure, plates are prepared according to the method of McLaughlin

et al. (10). To the freshly prepared plates aligned on the aligning tray are applied brisk vibrations generated from a Vortex Jr. mixer, through its round rubber adaptor, along the exposed edge of each plate. The vibrations redistribute the adsorbent slurry on the glass plate, thus smoothing out any uneveness resulting from the spreading process. This technique has been effectively used in the present investigations and appears to be an improvement over existing plate-preparation procedures.

SUMMARY

Preliminary studies on the influence of various carbon (sucrose, lactose, maltose, trehalose, fructose, glucose and galactose) and nitrogen (glycine, ammonia, nitrate and urea) sources on the growth of *P. baeocystis* as well as the production of psilocybin and its analogs by this fungus have been carried out. A quantitative TLC technique has been developed and used in these investigations. Results from these studies have indicated that psilocybin accumulation was highest in 2 weeks and decreased rapidly in 3 and 4 weeks. Psilocybin analogs were not produced by the fungus grown in any of the nitrogen sources when supplied singly.

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