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The identification of a germination factor for basidiospores of Psilocybe mutans

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THE IDENTIFICATION OF A GERMINATION FACTOR FOR BASIDIOSPORES OF PSILOCYBE MUTANS

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INTRODUCTION

Basidiospores of the coprophilous Basidiomycete Psilocybe mutans ined.[1] have been found to germinate extensively only in the presence of water extracts of animal dung (McKnight, 1956). McKnight found that 95% of the spores germinated in horse dung decoction, 94% in cow dung, 73% in pig dung, 28% in dog dung, and 26% in chicken dung decoctions. The decoctions were aqueous extracts of the dried dung. McKnight found that only 0.1 to 4.1% of the spores germinated in distilled water. Negative results were also obtained with 0.1% and 0.01% water solutions of malt extract, yeast extract, casein hydrolysate, and ribonucleic acid. Extracts of orange juice, of grass, and of alfalfa also gave negative results. Water solutions of furfural and hemoglobin resulted in 8.9% and 12.1% germination respectively. Spores treated in a water bath with temperatures ranging from 30°-60° C for periods of from 5 minutes to 5 hours did not germinate better than the water controls. However, 9.1% of the spores germinated when they were heated to 50° C for 15 minutes and then frozen for 24 hours.

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[1] This name was applied by Dr. Kent McKnight, USDA Crops Research Division, Beltsville, Maryland in an unpublished manuscript.

Dr. Dayna Stocks of the B.Y.U. Botany Department (unpublished data) has tested over 50 compounds for their ability to germinate the spores. All compounds tested gave negative results.

Psilocybe mutans is thus unique in that only water extracts of animal dung have, previous to this study, been found to cause germination. Most other fungal spores have been found to be stimulated to germinate by several different compounds or procedures (Sussman and Halvorson, 1966).

Other spores can be stimulated to germinate by dung decoctions; however, little work has been done to determine the factors in the dung that cause the spores to germinate. Cheo and Leach (1950) found that infusions of horse and cow dung were very effective agents in stimulating the spores of Ustilago striiformis to germinate. They showed that the stimulating factor was heat stable and that it was not a nutrient. One of their experiments indicated that the dung increased the permeability of the spore wall.

Dodge (1912) found that species of Ascobolaceae would germinate in a nutrient agar and dung decoction. Yu (1954) showed that Ascobolus species were activated to germinate by basic solutions in the presence of heat. For example, spores of A. magnificus germinated optimally in 0.19% NaOH at 37° C. Since Dodge (1912) had found the dung decoction to be quite basic, the high pH was probably

the factor in dung that stimulated the spores to germinate. Butler (1956) showed that spores of Sordaria fimicola were difficult to germinate except in the presence of a medium composed of beer wort and horse dung decoctions. However, he showed that the dung decoction could be replaced with a meat extract-tryptose-glucose agar at a pH between 4.5 and 5.0.

These few cases of increased germination by dung show that the influence of dung on the spore is probably unique to the respective kind of spore. This observation is consistent with the general rule that most spores seem to be different in their requirements for germination.

Identification of the specific factor in horse dung that activates P. mutans basidiospores to germinate will hopefully provide information concerning the germination process of this spore. Therefore the purpose of this study was to isolate and identify those compounds in horse dung that stimulate the basidiospores of P. mutans to germinate.

MATERIALS AND METHODS

Materials. Psilocybe mutans basidiospores were obtained from Dr. Dayna Stocks. Sodium cholate, sodium glycocholate, and sodium taurocholate were purchased from Nutritional Biochemical Corporation and the ox bile extract was purchased from Burroughs-Wellcome and Company. The DTAB was supplied and prepared by Dr. E.W. Anacker, Department of Chemistry, Montana State University.

Growth of basidiocarps. Psilocybe mutans was grown on a malt agar[1] (Brown, 1960) in a growth chamber at 22° C with 100 foot candles of continuous light. The Petri dishes were innoculated with 0.1 ml of germinating spores. The spores were germinated for about six hours prior to innoculation using a 1:1 mixture of a crude boiled water extract of horse dung and distilled water. After 12-16 days the basidiocarps were harvested and dried.

Assay for germination. The assay tested the ability of a compound or a mixture of compounds to germinate the

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[1] Malt extract 5.0 g
Maltose 5.0 g
$MgSO_4 \cdot 7H_2O$ 0.5 g
$Ca(NO_3)_2$ 0.5 g
$KHPO_4$ 0.25 g
Agar 25.0 g
Distilled H_2O 1.0 l

basidiospores of P. mutans. Two milliliters of the solution to be tested was placed in a small flask and autoclaved for at least twenty minutes at twenty pounds pressure. After cooling, a 0.1 ml suspension of spores was added to each flask. The suspension of spores was prepared by soaking 10-15 basidiocarps of P. mutans in approximately 10 ml of autoclaved distilled water. After squeezing the spores from the basidiocarps with a spatula, the basidiocarp fragments were filtered off through a fine mesh wire screen and the spores in suspension were concentrated by centrifugation. The assay flasks were then placed on a shaker and germinated at room temperature. The percent germination was determined by counting with a microscope. The counting was done in a random manner, but the same field was counted only once. The extended hyphae of the germinated spores often tended to clump together due to the continual agitation of the flasks. Small clumps were counted, but large clumps being difficult to count accurately, were not included in the germination percentages. Boiled water extract of the horse dung and distilled water were always assayed as controls with the other samples. The spores were considered germinated if the germ tubes were seen coming from the spores.

Extraction from horse dung. All studies were done using the same supply of horse dung. The horse dung was collected on September 27, 1966 by Dr. Dayna Stocks. Only

those pieces that were thoroughly dry were collected. The active factor was usually extracted from the horse dung with a ten fold excess (w/v) of boiling distilled water. The horse dung and water were boiled together for at least ten minutes, after which the undissolved solid material was removed by filtering through cotton. The resulting solution was centrifuged to spin down any remaining undissolved material. A variation of this method was tried with cold distilled water. A ten fold excess (w/v) of cold distilled water was stirred with horse dung for ten minutes and then filtered and centrifuged as above. The horse dung was also extracted with a fifteen fold excess of 10% sodium hydroxide. After stirring for about ten minutes the solid material was removed by filtering through Whatman #2 filter paper. Another method used was Soxhlet extraction with 95% ethanol for eight hours. A ten fold excess (w/v) of ethanol was used.

Purification of horse dung extracts. Once the active factor was dissolved, various methods were used to fractionate the resulting complex mixture. The following sequential procedures of fractionating the extracts were developed:

Procedure I: The boiled water extract of horse dung was dialyzed against at least a ten fold excess of distilled water in a refrigerator overnight. The solution that remained in the dialysis tubing was discarded, and

the solution in which the tube had been suspended was then reduced to about 50 ml using a Rinco flash evaporator. The pH of the resulting solution was adjusted to pH 2.0 with 1 N HCl. At this pH, a precipitate formed which was centrifuged off and discarded. The pH of the remaining solution was readjusted to pH 6.8 using 1 N NaOH. This solution was evaporated to dryness. The material which remained was partially dissolved in 50% ethanol. The residue was discarded and the ethanol solution was taken to dryness. The resulting residue was dissolved in a few milliliters of distilled water and stored for further use.

Procedure II: The boiled water extract of horse dung was concentrated to about one-fourth of its original volume using a Rinco flash evaporator. The horse dung extract was extracted five times with equal volumes of anhydrous diethyl ether. The ether was then allowed to evaporate, and the residue was extracted with water. Since very little of the residue was soluble in cold water, the residue-water mixture was heated on a hot plate for several minutes. The solution that resulted was saved for further use, and the residue was discarded.

Procedure III: Concentrated sulfuric acid was added to the sodium hydroxide extract of horse dung until a precipitate formed. The precipitate which formed at pH 2.0 was saved, and the remaining solution was discarded. The precipitate was dissolved in glacial acetic acid and the residue was discarded. The acid solution was diluted

with distilled water until a precipitate formed. This precipitate was collected and saved and the solution was discarded. The precipitate was dissolved in 95% ethanol. The residue that was not dissolved in the ethanol was discarded. The solution was evaporated to dryness. The residue resulting from evaporation was dissolved in boiling distilled water and it was saved. This residue was only partially soluble in distilled water.

Chromatography methods. Both gel filtration and thin layer chromatography were employed for further purification of horse dung extracts and the purification of the bile salt preparations. For the gel filtration, Sephadex G-10 was used in 2.5 X 45 cm columns. Five milliliters of sample was placed on the column and eluted with distilled water. The flow rate was set at 30 ml/hour at room temperature (25-30° C). The thin layer chromatography was done on 20 X 20 cm glass plates spread with Warner-Chilcott Laboratories silica gel G. The plates were spread to a thickness of 300 u and dried in the oven at 105° C for one hour. The plates were developed in glass chromatography chambers until the solvent front advanced 10 cm. Toluene-glacial acetic acid-water (10:10:1) (Sjovall, 1964) and ether-petroleum ether-methanol-glacial acetic acid (70:30:8:1) (Kritchevsky, Martak, and Rothblat, 1963) were used as solvent systems. Detection of organic compounds was accomplished by placing the dried developed

plates in a chamber with the atmosphere saturated with iodine vapor or by a spray composed of 0.5 ml anisaldehyde, 50 ml glacial acetic acid, and 1 ml of concentrated sulfuric acid. This reagent was prepared immediately before use. After spraying, the plates were heated in an oven at 125° C. The reagent is specific for most steroids (Kritchevsky, Martak, and Rothblat, 1963).

Removal of separated spots from thin layer plates. Once the thin layer plates had been developed as described above, the separated spots could be detected and removed from the plates. The spots on the plates were detected with iodine vapor. The plates were left in the iodine vapor only long enough for the spots to become faintly visible. The plates were then removed from the chamber. The plates were allowed to set until all the iodine color had left the plates before the spots were removed. The spots were marked and removed by scraping with a spatula. After they were removed, the spots were dissolved in 95% ethanol and centrifuged to remove the silica gel. The ethanol was evaporated and the residue was dissolved in 1 ml of water. The water solutions of each spot were assayed as described above.

Crystallization of cholic acid. Sodium cholate as supplied by Nutritional Biochemical Corporation is not chemically pure. The purity of the chemical was tested using thin layer chromatography, and the preparation was

found to contain at least two different compounds. Therefore, it was purified by crystallization. The sodium cholate was dissolved in glacial acetic acid. The acetic acid solution was diluted with distilled water until the solution became slightly cloudy. The solution was then placed in the refrigerator to allow the formation of cholic acid crystals. The cholic acid crystals were collected and washed with distilled water.

EXPERIMENTAL RESULTS

Solubility of active factor. Experiments were conducted to determine the solubility properties of the active factor. The results are presented in Table 1. These results show that the active factor is more soluble in ethanol than in the other solvents tested. When the horse dung which was first extracted with ethanol was then extracted with boiling water, no activity was found in the resultant water solution. This indicates that the active factor was completely dissolved by the ethanol. For the assay the ethanol was evaporated and the remaining residue was dissolved in water by refluxing. This water solution was then assayed. Something in the solution that resulted from refluxing the residue with water was inhibiting hyphal growth and germination. The inhibitor could be removed by centrifugation which indicates that its solubility in water is slight.

On the basis of percent germination, the boiled water extract was more successful than the other solvents in dissolving the active factor sufficiently to stimulate spore germination. However, Stocks (unpublished data) found that the active factor is only slightly soluble in boiling water. After extracting 25 g of horse dung with

Table 1. Assay of procedures for extracting the active factor from horse dung.

Procedure	% Germination[1]	Comments
Boiled water extract	97	Extensive clumping
Cold water extract	5	
Ether extract of boiled water extract[2]	61	
Boiled water extract after extraction with ether	60	
Soxhlet extraction with 95% ethanol[3]	11	Hyphal growth inhibited
Water solution of ethanol extract after centrifugation[4]	50	
Boiled water extract of the horse dung that had been extracted with ethanol	0	
10% sodium hydroxide extraction[5]	43	Hyphal growth inhibited

[1]Spores were germinated 18 hours. The percentage was based on 100 spores counted. The spores were six months old.

[2]After extraction, the ether was evaporated and the residue dissolved in water for the assay (purification procedure II).

[3]After the ethanol extraction, the ethanol was evaporated off. The residue was refluxed with water to dissolve the residue in the water. A considerable amount of material remained in suspension in the assay solution.

[4]Centrifugation of the water solution of the Soxhlet extraction (footnote 3).

[5]Purification procedure III.

250 ml of boiling water ten times in sucession, he found that after three hours the first extract stimulated 46% germination and the tenth extract stimulated 14% germination. The first five extracts were all about the same in their ability to stimulate spore germination.

Since the extraction of a volume of boiled water extract of horse dung with four times its volume of diethyl ether did not substantially reduce activity in the solution extracted (boiled water extract), this indicates that the active factor was only slightly soluble in ether.

Although not strictly comparable to the above experiments, the results of purification procedure III indicate that the active factor is soluble in 10% NaOH. This solution was used to make the initial extract from the horse dung in this purification procedure. However, the solubility of the active factor in the sodium hydroxide solution cannot be determined from this experiment.

Approximate molecular weight. An approximate molecular weight of the active factor can be determined by gel filtration. Sephadex G-10 excludes all molecules above the molecular weight of 700. Horse dung extract was purified by procedure I. Five milliliters of this extract was eluted with water through a column of Sephadex G-10. After 40 ml was eluted every 20 ml was concentrated by flash evaporation to about 2 ml. The combined fractions were then assayed. The results of the assay are recorded

in Table 2. Most of the pigment of the horse dung came off the column after 125 ml was eluted. Thus, since the activity came off after 180 ml was eluted (Table 2), the active factor was held up on the column. It thus has a molecular weight of less than 700.

Miscellaneous properties of the active factor. During the experiments various properties of the active factor became apparent. The factor is very heat stable. The horse dung extract was autoclaved at pH 6.8 several times without altering the ability of the factor to germinate spores. However, refluxing the boiled horse dung extract with 1 N KOH overnight did destroy the ability of the factor to germinate the spores.

The behavior of the factor in acid solutions was erratic. When the pH of the dialyzed boiled water extract of horse dung was lowered to 2.0, both the resultant precipitate and the supernatant were able to stimulate over 50% germination. The precipitate was redissolved in 1 N NaOH and the pH was adjusted to 6.8 for the assay. Table 3 shows the results of an experiment which was designed to determine whether the activity could be removed from the solution by repeated acid precipitations. Previously it had been observed that even after centrifuging the acid precipitate, if the solution was allowed to stand under low pH conditions, a new precipitate would form. In addition freezing of the acid solution increased the precip-

Table 2. Assay of fractions from Sephadex G-10 column.

Ml. eluted	% Germination	Comments
40-60	1	
60-80	1	
80-100	0	
100-115	1	
115-135	1	
135-155	1	
155-180	15	
180-200	65	Extensive clumping
200-220	70	Extensive clumping
220-240	50	Little clumping
240-260	7	
260-280	3	
280-300	1	

[1]The spores were germinated 17 hours. The percentage was based on a total count of 100 spores. The spores were 3 months old.

Table 3. Assay of acid precipitates of horse dung extract.

Procedure[1]	% Germination[2]	Comments
Acid ppt 1	42	
Acid ppt 2	1	
Frozen acid ppt 3*	95	Clumping
Frozen acid ppt 4	0	
Frozen acid ppt 5	0	
Frozen acid ppt 6	65	Clumping
Frozen acid ppt 7	0	
Frozen acid ppt 8	29	
Frozen acid ppt 9	80	Clumping
Solution after acid precipitations	0	

[1]For the assay, the precipitate was dissolved in 1 N NaOH. The pH was then adjusted to 6.8. All 9 precipitations were made from the same 20 ml of horse dung extract.

[2]The spores were germinated 18 hours. The percentage is based on a total count of 100 spores. The spores were 3 months old.

*After the pH was lowered to 2.0, these solutions were frozen at least overnight after which the precipitate was removed and assayed.

itate formation. Thus, the pH of about 20 ml of boiled water extract was lowered to 2.0 with 1 N HCl. The precipitate was removed and assayed as described above. This procedure was carried out nine times on the same solution. The first two times the precipitate was removed the solution was not frozen, while the other times the precipitate was removed, it was frozen in acid form overnight or longer. The results (Table 3) show that after the nine precipitations, all activity was lost from the solution. The pattern of activity found in the precipitates gave no clues concerning the conditions necessary for the active factor to be precipitated.

Activity range determination. To determine in what concentration range the active factor would germinate the spores, horse dung, purified by procedure I and run through a Sephadex G-10 column for further purification, was collected, weighed, and assayed. Twenty milliliter aliquots of the fractions between 145 and 220 ml eluted were evaporated to dryness, weighed in a previously tared container, and completely dissolved in 1 ml of water for assay. In addition, ten fold and one hundred fold dilutions were made of each sample and these samples were assayed. Table 4 gives the results of this experiment. The results indicate that diluting the active factor ten fold results in a concentration unable to stimulate germination. This concentration of the active factor is 100 ppm or less

Table 4. Activity range of active factor.

Mls eluted[1]	Wt. of residue	Conc. of Assay soln.	% Germination[2]
140-160	1.062 mg	1062 ppm	60
10X dilution		106 ppm	0
100X dilution		11 ppm	0
160-180	0.795 mg	795 ppm	4
10X dilution		80 ppm	0
100X dilution		8 ppm	0
180-200	0.600 mg	600 ppm	3
10X dilution		60 ppm	0
100X dilution		6 ppm	0
200-220	0.570 mg	570 ppm	35
10X dilution		57 ppm	0
100X dilution		6 ppm	0

[1]The sample was purified by running through a Sephadex G-10 column. The fractions indicated were combined and evaporated to dryness. The residue was dissolved in 1 ml of water for the assay.

[2]The spores were germinated 14 hours. The percentage was based on a total count of 100 spores. The spores were 3 months old.

depending upon the purity of the residue assayed. Since the residue was not crystalline, it evidently was not very pure.

Assay of bile components. Since the solubility properties of the active factor were found to be similar to the bile acids, bile extract and some of the components of bile were assayed. Table 5 lists the compounds that were assayed. The only two substances tested that had significant activity were ox bile extract and a preparation of sodium glycocholate. Chromatography of the sodium glycocholate preparation, using the toluene-glacial acetic acid-water solvent system on a thin layer plate, showed that the preparation was quite impure. There were nine spots which were visible with the iodine vapor, most of which corresponded to spots present in a similar chromatogram of the ox bile extract. Using the anisaldehyde detection reagent, seven of the nine spots which were detected by the iodine vapor, were visible. Detection by anisaldehyde indicates that these seven spots were steroid compounds. They were probably cholesterol and various bile acids because these are the only steroids present in bile. It was observed (Table 5) that the sodium glycocholate preparation had an optimum concentration for stimulating germination.

Assay of components of the sodium glycocholate preparation. Thin layer chromatography plates were spotted

Table 5. Assay of the common compounds found in bile.

Compounds assayed	% Germination[1]	Comments
Ox bile extract		
33 mg/ml	35	
Sodium glycocholate		
0.1 mg/ml	0	
0.275 "	4	
0.55 "	76	Extensive clumping
1.7 "	30	Hyphal growth inhibited
3.3 "	17	Hyphal growth inhibited
6.7 "	1	
15.0 "	0	
33.5 "	0	
Sodium taurocholate		
0.2 mg/ml	1	
0.35 "	0	
0.425 "	0	
0.50 "	3	
1.05 "	2	
1.82 "	1	
2.0 "	1	
3.55 "	0	
5.0 "	1	
Sodium cholate		
0.005 mg/ml	0	
0.001 "	3	
0.05 "	0	
0.01 "	1	
0.025 "	2	
0.05 "	2	
0.075 "	5	
0.1 "	1	
0.6 "	0	
1.5 "	0	
4.35 "	2	
5.5 "	1	

[1]The spores were germinated 18 hours. The percentage was based on a total count of 100 spores. The spores were 3 months old.

Table 5 (continued)

Compounds assayed	% Germination	Comments
Lecithin		
0.5 mg/ml	0	
1.0 "	0	
5.0 "	0	
10.0 "	0	
Choline-HCl		
0.5 mg/ml	1	
1.0 "	0	
5.0 "	0	
10.0 "	0	

with 0.8 mg, 1.3 mg, and 2.6 mg of sodium glycocholate respectively and developed in the toluene-glacial acetic acid-water solvent system. Two separate runs were made with plates spotted with 1.3 mg. The spots which developed were removed and assayed according to the previously described procedure. Table 6 shows the results of the assay. Co-chromatography of the sodium glycocholate was performed with crystallized cholic acid. The results of the co-chromatography showed that the spot with the Rf value of 0.65 was cholic acid. Since the results of the assay of this spot were somewhat conflicting, the above experiment was repeated with modifications. Both sodium cholate (0.2 mg) and sodium glycocholate (1.3 mg) were spotted on thin layer chromatography plates. The plates were developed in the toluene-acetic acid-water solvent system. Only the spots with Rf values of 0.65 were removed and assayed as above. The results are recorded in

Table 6. Assay of sodium glycocholate spots separated by toluene-glacial acetic acid-water (10:10:1).

Rf value	% Germination[1]			
	0.8 mg[2]	1.3 mg	1.3 mg	2.6 mg
0	2	1	5	0
0.09	7	0	2	0
0.18	6	12	5	11
0.33	24	0	5	6
0.48	3	1	0	5
0.65	5	0	50*	13
0.85	11	1	0	11
0.91	0	0		1
0.97	0	0	3	4

[1]The spores were germinated 18 hours. The percentage was based on a total count of 100 spores. The spores were 1 year old.

[2]The total number of mg spotted on the thin layer plate.

*Due to clumping no count was taken. There was greater than 50% germination.

Table 7. To confirm the results, a second solvent system, ether-petroleum ether-methanol-acetic acid (70:30:8:1), was used. Again both sodium cholate (0.2 mg) and sodium glycocholate (1.3 mg) were spotted on the plates. The plates were developed and the spots with Rf values of 0.3 were removed (cholic acid), and assayed. The results of this experiment are also recorded in Table 7. These experiments show that cholic acid does stimulate the spores to germinate. The results (Table 6) show, however, that cholate is not the only compound that stimulates germination. The spot with the Rf value of 0.33 also had significant activity. The other spots showing activity could be the result of incomplete separation of these spots from those with Rf values of 0.33 and 0.65. All spots, however, appeared to be separated. If they were separated, at least four spots showed significant activity, i.e., Rf 0.18, 0.33, 0.65, and 0.85. All four of these spots were visible under the anisaldehyde reagent and are therefore steroids. The concentration of the compounds (Table 6) appears to be critical in determining their ability to germinate the spores.

Assay of purified cholic acid. Since the sodium cholate preparation used was not pure it was purified by changing it to the acid form and crystallizing. Chromatography on thin layer plates showed that a slight impurity remained with the cholic acid after crystallization.

Table 7. Assay of cholate spots.

Preparation spotted[1]	Spot assayed	% Germination[2]	Comments
Separated by toluene-acetic acid-water (10:10:1)			
Sodium cholate	Rf 0.65	14	Clumping[3]
Sodium glycocholate	Rf 0.65	18	Clumping
Separated by ether-petroleum ether-methanol-acetic acid (70:30:8:1)			
Sodium cholate	Rf 0.3	10	Clumping
Sodium glycocholate	Rf 0.3	23	Clumping

[1]0.2 mg of sodium cholate was spotted and 1.3 mg of sodium glycocholate was spotted in each case.

[2]The spores were germinated 16 hours. The percentage was based on a total count of 200 spores. The spores were 1 year old.

[3]In all cases the spores in the clumps were not counted, thus the actual germination percentage is higher than indicated.

Table 8 gives the results of the assay of various concentrations of cholic acid. As can be seen, the activity is quite low.

Table 8. Assay of crystallized cholic acid.

Concentration	% Germination[1]
0.04 mg/ml	0.5
0.06 "	7
0.08 "	2.5
0.1 "	0
0.3 "	0
0.5 "	0

[1]The spores were germinated for 10 hours. The percentage was based on a total count of 200 spores. The spores were 1 year old.

Assay of surface active agents. In higher animals the various cholate derivatives in bile function as surface active agents in the digestion of lipids. To determine whether a general surface active agent could stimulate germination, two surface active agents, "Ivory Liquid" dish soap and dodecyltrimethylammonium bromide, were assayed. A 0.5% (w/v) solution of "Ivory Liquid" was made. The dodecyltrimethylammonium bromide (DTAB) solution consisted of 0.5496 g NaBr, 0.0406 g DTAB, and 9.8367 g of water. The assay was run by putting the spores in the respective detergents and then an aliquot of spores was withdrawn from the detergent after increasing lengths of time. The amount of time the spores were in the detergents varied from 5 minutes to 18 hours. The spores were centrifuged and removed from the detergent,

and then washed three times in distilled water. The spores were then placed in distilled water for the remainder of the 18 hour germination period. The results in all cases were negative.

Purification of active factor of horse dung. Since the assay of cholic acid and its solubility properties indicate that it is possibly the active factor in horse dung, a purification procedure for the active factor was designed based on the properties of cholic acid. Purification procedure III was developed as the result. The active factor in horse dung was purified using this procedure. At the stage where the horse dung was dissolved in ethanol, a thin layer plate was run using the toluene-acetic acid-water solvent system. One spot detected by iodine vapor had the same Rf value as the cholic acid (0.65). After the ethanol was evaporated, the residue was dissolved in 2 ml of hot water. However, only a small portion of the residue was dissolved in the water. An assay was run on the solution after the remaining residue was removed. After 10 hours, the purified horse dung solution stimulated 9.5% of the spores to germinate. After 18 hours, 24% of the spores had germinated. Although the spores germinated, none of the spores developed germ tubes longer than one-half the length of the spores. Evidently something inhibited hyphal growth and perhaps even germination. After assaying the solution, a second attempt was made to dissolve the remainder of the ethanol residue

in hot water. However, again the residue was only slightly soluble in the hot water. A small amount of the water solution was spotted on a thin layer plate and developed in the toluene-acetic acid-water solvent system. This time the spot with the same Rf value as cholic acid was missing. Several other spots were present which under iodine vapor had the same Rf values as some of the components of bile. A second chromatogram of this same solution was detected with the anisaldehyde reagent. This method did not reveal any spots. Thus, this second solution of the ethanol residue does not contain steroids. An assay was run on this second solution. The activity was much greater than the first hot water extract of the ethanol residue. After 21 hours, 43% of the spores had germinated. Many large clumps were present that were not counted; thus the actural germination is greater than 43%. Again something appeared to be inhibiting hyphal growth since less than 10% of the germinated spores had germ tubes longer than one-half the length of the spores. This assay also showed that something in the first hot water extract of the ethanol residue was inhibiting spore germination, since the first assay had a much lower germination percentage than the second under similar conditions.

DISCUSSION

Basidiospores of Psilocybe mutans tend to gradually decrease in their ability to germinate as they become old. While no specific tests were made to compare fresh spores with older spores it has been observed that one year old spores tend to germinate only about 60% in the boiled water extract of horse dung while fresh spores germinate over 90% in this solution. The distilled water controls always germinate less than 3%.

Speculations can be made concerning the molecular structure of the active factor in horse dung. The molecular weight approximations indicate that the compound has a molecular weight of less than 700. Since it is soluble in 10% NaOH, and is precipitated by acid solutions, this indicates that it probably has a carboxylic acid group. Its limited solubility in water would indicate that there are at least six or more non-polar carbons for each polar functional group. The low water solubility would also eliminate the possibility that the compound is in the salt form of the acid. Its low solubility in ether is evidence that it is not just a low molecular weight compound with one functional group. Hence, the compound probably has a relatively high molecular weight (less than 700) with

several functional groups. Such a compound would be quite soluble in alcohol, which is a property of the active factor. The solubility in NaOH and the low solubility in acid eliminates the possibility that the compound has basic properties.

The compound is very stable since autoclaving it repeatedly does not alter its activity. However, it does have some bond that can be hydrolyzed, since activity was destroyed by refluxing it with 1 N KOH overnight. This could indicate the presence of a peptide, a glycoside, or an ester bond.

The properties of the active factor in acid solutions hint that there is more than one active factor. A single compound would not be expected to have such an erratic pattern of activity in the precipitates. However, if more than one active factor is present, each with an acid group, but slightly different in structure, the results would not be too difficult to explain.

The possibility that more than one active factor is present is supported by the results of the assay of the components of the sodium glycocholate preparation. Although the results of this experiment are not really consistent, they do indicate that at different concentrations, different spots are active. Thus there is a strong possibility that more than one compound is able to stimulate the spores to germinate. The fact that there is such a variation in the spots showing germination activity

is probably due to the problem of controlling the concentration. Since several steps are involved in the process of removing the spots from the plates, controlling the concentration of one spot in relation to the others is difficult.

The spot that showed the greatest activity in Table 6 was cholic acid. That cholic acid does stimulate the spores to germinate is corroborated by the results recorded in Table 7. Some of the other spots that showed activity are undoubtably also bile acids. The four spots showing the greatest activity were all steroids, and thus probably bile acids.

The fact that the crystallized cholic acid showed an activity of only 7% is difficult to explain. It is possible that even with the crystallization, the impurities that remained with the cholic acid are inhibitory to germination. It is also possible that cholic acid alone is not sufficient to cause germination; that some compound, not effectively separated from the cholate on the thin layer plates, is essential for the germination of the spores.

There is some evidence to indicate that cholic acid or some form of this compound is one of the active factors in horse dung. Cholic acid has a molecular weight of 409, and it has solubilities of 0.28 g/l at 15^{o} C in water, 1.22 g/l in ether, and 30.56 g/l in alcohol. It has one carboxylic acid group and three alcohol groups. Most of

the other bile acids have solubility properties that are very similar to cholic acid and they have molecular weights of from approximately 400 to 475. These compounds thus have essentially the same properties as those known for the active factor in horse dung.

The possibility that the bile acids are the active factor in horse dung is supported by the fact that bile acids have been found in dung. Most of the work has been done on the identification of the bile acids in human feces, however, the same bile acids are found in horse bile as in human bile (Fieser and Fieser, 1959), thus there is no reason to assume that the bile acids found in human feces would be significantly different than those in horse dung. Danielsson, et. al., (1963) reported the presence of deoxycholic, lithocholic, and cholic acid in human feces. Other bile acids have been found in human feces by Eneroth, Gordon, and Sjovall (1966).

The ability of the sodium glycocholate preparation to stimulate germination is related to the concentration of this bile salt preparation. The optimum germination occured at a concentration of 0.55 mg/ml. It is probably more than a coincidence that the critical micellar concentration (CMC) of a mixture of bile salts like that of the sodium glycocholate preparation is about 1 mM or approximately 0.45 mg/ml (Hofmann and Small, 1967). Since, with the formation of the micelles the solubilization properties of the bile salts change abruptly, this change in their

ability to solubilize lipids is possibly related to their stimulation of germination. It is noted that at concentrations below this optimum concentration of 0.55 mg/ml, the ability of the bile salts to stimulate germination decreases abruptly. This would correspond with the difference in the solubilization properties of the bile salts below the CMC as compared to above the CMC. The decreasing ability of the bile salts to stimulate germination at concentrations much above the CMC is probably due to the observed inhibition of hyphal growth and germination at the higher concentrations of bile salts. Either the bile salts or some impurity are inhibiting the growth and germination.

The inhibition of growth and germination has been observed, not only with the bile salt preparations, but also with two of the horse dung extracts. Significantly, these are the two extraction methods that dissolve the greatest amounts of bile acids, i.e., ethanol extraction and purification procedure III. This could indicate that the horse dung extracts are inhibiting the growth and germination of the spores by processes similar to those of the bile salts.

It is interesting to note that since the bile acids are possibly present in their acid rather than salt form in dung, this prevents the bile acids from being dissolved in water extracts of the dung at concentrations that would inhibit germination. Since they are extracted with boil-

ing water, the concentration of the bile acids in the water, after it has cooled, would probably be close to their saturation levels. This concentration is about the same concentration as the CMC of the bile salts. This is only an approximation however, since the CMC is dependant on the Na^+ concentration, which is unknown for these solutions.

The fact that the surface active agents tested were not able to stimulate germination does not rule out the possibility that the bile salts act as surface active agents in stimulating germination. The surface active properties of the detergents tested are quite different from those of the bile salts (Hofmann and Small, 1967).

More work needs to be done to show that the bile salts do stimulate germination due to their surface active properties. It must also be shown that the bile acids in the dung are responsible for the germination of the basidiospores.

LITERATURE CITED

Brown, R. C. 1960. A study of the effects of different substrates on growth and reproduction on certain coprophilic Agaricaceae. Unpublished thesis. Brigham Young University. Provo, Utah.

Butler, E. E. 1956. Spore germination in Sordaria fimicola. Mycologia 48:345-348.

Cheo, P. and J. G. Leach. 1950. The stimulating effect of dung infusion on the germination of spores of Ustilago striiformis. Phytopathology 40:584-589.

Danielsson, H., P. Eneroth, K. Hellstrom, S. Linstedt, and J. Sjovall. 1963. On the turnover and excretory products of cholic and chenodeoxycholic acid in man. J. Biol. Chem. 238:2299-2234.

Dodge, B. O. 1912. Methods of culture and the morphology of the archicarp in certain species of the Ascobolaceae. Bull. Torrey Bot. Club 39:139-197.

Eneroth, P., B. Gordon, and J. Sjovall. 1966. Characterization of trisubstituted cholanoic acids in human feces. J. Lipid Res. 7:524-530.

Fieser, L. F. and M. Fieser. 1959. Steroids. Reinhold Publishing Corp., New York.

Hofmann, A. F. and D. M. Small. 1967. Detergent proper-

ties of bile salts, pp. 333-376. In A. C. DeGraff and W. P. Creger (ed.) Annual Review of Medicine, vol. 18. Annual Reviews, Inc., Palo Alto, Calif.

Kritchevsky, D., Martak, D. S., and G. H. Rothblat. 1963. Detection of bile acids in thin-layer chromatography. Analyt. Biochem. 5:388-391.

McKnight, K. H. 1956. Certain factors affecting spore germination in Psilocybe mutans. Proc. Utah Acad. Arts, Sci., and Letters 33:177-178.

Sjovall, J. 1964. Separation and determination of bile acids, pp. 97-141. In D. Glick (ed.) Methods of Biochemical Analysis, vol. 12. Interscience Publishers, New York, London, and Sydney.

Sussman, A. S. and H. O. Halvorson. 1966. Spores, their dormancy and germination. Harper & Row, Publishers, New York and London.

Yu, C. C. 1954. The culture and spore germination of Ascobolus with emphasis on A. magnificus. Am. J. Botany 41:21-30.

ABSTRACT

Basidiospores of the Basidiomycete Psilocybe mutans have been found to germinate only in the presence of water extracts of animal dung. The chemical nature of the factor present in the dung that induces germination is not known, nor has it ever been isolated.

Experiments showed that extracts of bile salts cause the basidiospores to germinate, indicating that since bile salts are found in dung, they may be the factor which stimulates the spores to germinate. Investigations of the properties of the factor in dung show that the factor has solubility properties that are similar to those of the bile acids.

It was found that the bile salts are able to stimulate the germination of the spores optimally at a concentration that corresponds to the critical micellar concentration of the bile salts. This suggests that the bile salts activate the germination due to their surface active properties.