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Revival of Metabolism in Rehydrated *Marasmius oreades*

Lorie J. Warren
College of Saint Benedict/Saint John's University

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Revival of Metabolism in Rehydrated *Marasmius oreades*

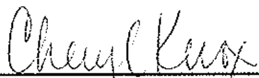
A Thesis
The Honors Program
College of St. Benedict/St. John's University

In Partial Fulfillment
of the requirements for the Distinction of "All College Honors"
and the Degree Bachelor of Arts
In the Department of Biology

by
Lorie J. Warren
April, 1992

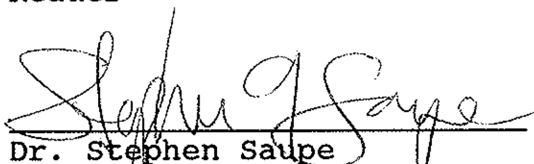
Approval Page
Lorie Warren

Dr. Margaret L. Cook
Director of Honors



Dr. Cheryl A. Knox
Assistant Professor of Biology
Reader

Dr. Nick Zaczkowski
Professor of Biology
Reader



Dr. Stephen Saupe
Associate Professor of Biology
Advisor

Dr. Charles F. Rodell
Chair of Biology Department

Table of Contents

Introduction	Page 6
Methods and materials	Page 11
Results	Page 12
Discussion	Page 14
Tables and Graphs	Page 17
Works cited	Page 34

INTRODUCTION

In the mushroom family Tricholomataceae most species of the genus *Marasmius* and a few in the genus *Collybia* are able to revive (Gilliam 1976). Mushrooms that revive spring back to their original forms when moistened after dehydration. This characteristic distinguishes *Marasmius* from the closely related genus *Collybia* (Gilliam 1976).

Marasmius spp. are generally small and brown with white spores. They inhabit mainly woodland areas but some, such as the well known fairy ring mushroom (*Marasmius oreades*(Bolt ex Fr) Fr), prefer open grasslands and lawns. Depending on the species, fruiting may occur from mid June through October in moist conditions.

These mushrooms may suddenly appear after or during rain showers. In reality they may have already been present, shriveled and dry, waiting for moist conditions to rehydrate, revive, and release spores (Arora 1979). Since moist conditions are needed for the survival of the spores, the ability of the fruiting bodies to dry out and revive extends the sporing period.

The reason that *Marasmius* spp. can revive while other species do not is not currently known. However, anhydrobiosis in animals and plants may provide some answers.

Anhydrobiosis is a type of dormancy characterized by very little or no metabolic activity. It is induced by the removal of water from the organism (Keilin 1959). While the dessicated organisms appear shrunken and lifeless they can quickly swell back to

their normal shape and resume metabolic activity when wetted (Crowe 1971).

The study of anhydrobiosis began in 1702 with Antony Von Leewenhoek (Keilin 1959). He discovered tiny animals (now known as rotifers) in the dry rain gutters of his house. These animals would suddenly come to life when water was added to them. Since that time scientists have discovered several other multicellular organisms such as rotifers (Weisburd 1988), tardigrades (Young 1985), brine shrimp (Crowe, et al 1984), and nematodes (Crowe 1971) that have the ability to withstand long periods of drying.

Species that exhibit anhydrobiosis often inhabit hostile and unpredictable environments such as areas that often dry out or freeze (Rensberger 1980). Anhydrobiosis gives these animals added resistance to environmental extremes that would otherwise kill them in their hydrated state (Young 1985). Rotifers, nematodes, brine shrimp, and tardigrades have been kept at a few degrees Kelvin for several days and remained viable (Morowitz 1979). Dessicated tardigrades and rotifers can withstand temperatures of 151 C for as long as 15 minutes (Young 1985). Dried nematodes have survived 48 hours in a vacuum of 4 or 5 mm of mercury (Rensberger 1980) and they have a high tolerance for x-rays, about 1000 times that of a human (Young 1985).

Anhydrobiosis increases the life span of the organism. The one year life span of a tardigrade, for example can last up to 60 years with frequent drying periods (Young 1985). The record seems to go to some tardigrades and rotifers stored for 120 years in an old moss herbarium specimen. When the moss was moistened they revived although they died soon after. Death was probably caused by a build up of free radicals (Weisburd 1988),

that are deleterious chemicals formed when oxygen reacts with the molecules of the dried organism (Rensberger 1980). Free radicals can do extensive damage to the membranes (Weisburd 1988). Organisms stored without oxygen may have a much higher survival rate because they are not being exposed to the dangerous build-up of free radicals. However, there is no answer to how long the organisms can survive in the dehydrated state.

Most organisms are 50-90% water. Humans will die with the loss of just one-fifth of their water content (Young 1985). A tardigrade, however, can be dried to a water content of 2% its body weight (Weisburd 1988). It is unlikely that any metabolic activity can take place in the dried organisms because metabolic activity is presumed to require aqueous solutions (Clegg 1973). The small amount of water that is present is immobile and incapable of hydrating all the proteins and intracellular organelles (Clegg 1973). On the average it requires 0.25-0.75g of water to bond with one gram of protein in the hydrated state (Roser 1991). It seems very likely that while in the dry state anhydrobiotic organisms do not metabolize.

Water plays an important role in the structure of the macromolecules of the cell (Clegg 1973). Water molecules form a hydrogen-bonded network that separates the phospholipid's phosphate head groups from one another (Weisburd 1988). When water leaves the cell, membranes are highly susceptible to damage (Young 1985). Membranes may fuse together or, a redistribution of intermembrane particles may occur (Crowe, et al 1984). This structural and functional damage is severe and irreparable.

The sugar trehalose has been proposed as a substance that bonds with the polar head groups of the membranes to act as a

water replacement (Crowe, et al 1984). Trehalose is a 1-1 glucose disaccharide which is soluble in water. It is a non-reducing sugar and therefore does not interfere with the enzymatic activity of cells. It is capable of forming hydrogen bonds with macromolecular structures (Roser 1991).

Trehalose is very common in anhydrobiotic organisms. It may make up 20% of the dry weight of brine shrimp cysts, nematodes, dry active bakers yeast, and spores of certain fungi (Crowe, et al 1984). Nematode survival during dessication has been linked to trehalose (Crowe, et al 1987).

Yeast, fungal spores, macrocysts of slime mold and some seeds of higher plants also exhibit anhydrobiosis (Marino 1989). Trehalose is very common in these organisms too (Elbein 1974). Dry active bakers yeast for instance, has been found to have trehalose levels of 10-18% (Pollock, et al 1951). Further, high trehalose levels have been been linked to good gassing ability after rehydration (Pollock, et. al. 1951). In fact mutant strains of yeast unable to synthesize trehalose do not survive dessication (Roser 1991).

Trehalose levels in yeast increase during drying (Pollock et al 1951). Water loss serves as an activator for trehalose synthesis (Marino 1989). The breaking of dormancy also effects the amount of trehalose in fungal spores. An increase in trehalase, the enzyme that breaks down trehalose, can be measured (Thevelein 1983).

In light of the role of trehalose in the reviving capabilities of yeast and other organisms I hypothesized that trehalose was the key factor in the ability of <u>Marasmius</u> to revive after dessication. I was also interested in the ability

of mushrooms to metabolize after revival and not just act as a sponge taking up water. I tested the revival capabilities of Marasmius and other mushrooms and compared those results with information about trehalose content found in the literature. It was my hope to find a correlation between trehalose content and the ability to revive.

Methods and Materials

A. Tissue preparation

Mushrooms were collected in Stearns County Minnesota during September and October or obtained from the CSB/SJU Biology Department herbarium (CSB). They were air dried and stored in wax bags at room temperature. Voucher specimens of all specimens are at CSB. Unless otherwise specified all trials and experiments were done using *Marasmius oreades*(Bolt ex Fr) Fr.

B. Measuring metabolism after revival

The method I developed to measure metabolism after revival was adapted from an article by Becwar et al (1982) concerning the revival of seeds. This assay is based on the ability of living tissue to reduce 2-3-5-triphenyltetrazolium chloride is reduced from a clear solution to an insoluable red triphenylformazan pigment by dehydrogenase activity. Dehydrogenases transfer electrons or hydrogen atoms to coenzymes, such as NADH or NADP, from metabolic intermediates during aerobic metabolism. These coenzymes transfer electrons to oxygen and ultimately ATP is produced. The dehydrogenases are also able to transfer the electrons to the TTC salts, reducing them. In this

way the amount of formazan pigment can be an indicator of the dehydrogenase activity which occurs only in live cells (Ross 1974).

Mushroom samples were weighed and homogenized in a 0.2M sodium phosphate buffer (pH 7) with a Polytron homogenizer or a morter and pestle. The homogenate (2 ml) was added to 3ml of a 1% w/v solution of triphenyltetrazolium chloride (TTC). The mixture was incubated for 4-10 hours (later experiments were 10 hours) in a shaker bath shaking on medium speed at 32°C, and then centrifuged for five minutes. The supernatant was decanted and the pellet was resuspended in 100% Methanol. This mixture was incubated in a water bath for two hours at 55°C to extract the water insoluble red formazan pigment from the tissue. The samples were allowed to cool and the absorbance was measured at 530 nm with a Beckman spectrometer.

C. Standardized curve

I made a stock solution of 0.7mg NADH in 5ml of the 0.2M sodium phosphate buffer. This was mixed with the 1% TTC solution in varying proportions.

RESULTS

I began my investigation by developing a method to test for metabolism of *Marasmius* after revival. Although the basic method was adapted from Becwar et al (1981), much of my time was spent adapting the method for use with mushrooms.

The first step was to find the maximum absorbance of the formazan pigment created by the reduction of the TTC. I wanted a wavelength that would discriminate between high and low levels

of formazan pigment. I ran absorption spectra of two samples. The first was a sample that contained an abundance of formazan pigment and the second was a control in which the TTC was absent (Fig 1 and 2). The formazan absorbance ranged from about 400-550nm, with the maximum at 480nm. The control absorbed little above 400nm. It's highest absorption was 340nm. Thus I selected an absorbance of 530nm to use for my experiments.

I also determined the best medium for extracting the formazan pigment. Mushroom samples (5 ml) were extracted in either 95% ethanol or 100% methanol and their absorbances were measured (Table 1). Although the ethanol samples had higher absorbance values than the methanol extracted tissues, the former samples were cloudy. The higher absorbance may have been due to the cloudy nature of the samples because, to the naked eye they were no redder then the methanol samples. Because of the clarity of the extracts I used methanol as the extraction agent.

An experiment was run to determine the appropriate length of time to incubate the rehydrated samples with TTC. Samples were treated with TTC for various times, the color extracted with MEOH, and absorbance measured (Fig 3). The minimum required time for incubation for the mushrooms in the TTC was about three hours. Longer incubation times resulted in greater formazan production. However, times were limited to 10 hours to reduce the risk of contamination from microbes.

To demonstrate that formazan pigment formation was due to enzymatic activity in the rehydrated mushrooms, I attempted to kill activity by microwaving dry and rehydrated samples for various times. Microwaving rehydrated samples for one minute decreased activity by 50% (Fig 4). In a preliminary experiment,

dry samples remained active even after five minutes of microwaving.

Herbarium samples of *M. oreades* that had been stored for 18-40 months were studied to determine the effect of duration of dry storage on metabolic revival (Fig 5). There was little, if any, correlation between the age of the samples and their absorbance (corr. coeff.=0.003). Thus, up to 40 months of dry storage made little difference on rehydration of metabolism.

I attempted to quantify the amount of TTC reduction in *M. oreades* by using NADH. The theory was that by adding a known amount of NADH to reduce TTC we could create a standard curve of the amount of pigment reduced. Unfortunately, the TTC was not reduced by the NADH alone and no response was observed. So the method was abandoned.

A literature search failed to support my hypothesis that there would be a correlation between trehalose content and revival ability (Tables 2 and 3). I found 80 spp. of mushrooms. 32 had trehalose ranging from trace amounts to 3.5% in *Lactarius piperatus*. The only mushroom known to spring back to it's original shape on the list is *M. oreades* with a trehalose level of 0.35%.

Discussion

Fresh mushrooms stained red with the TTC staining technique. Dried/rehydrated *Marasmius* mushrooms also produced the pigment when incubated with TTC. Upon reviving *Marasmius oreades* does metabolize.

Microwaving stopped the activity of the rehydrated

mushrooms, they did not reduce the TTC. Thus the formation of the pigment is an active process that requires enzymes and metabolism and is associated with life processes.

Microbes were probably not responsible for the formation of the pigment. The incubation times were kept short to limit microbial contamination. As a second precaution, the TTC solution was drained away from the samples and they were rinsed with fresh sodium phosphate buffer to ensure that the only color present was that extracted from the tissues.

The length of time that the samples were dry was not correlated to a decrease in metabolic activity. Longevity in a fungus has been noted previously; sporophores of the *Schizophyllum commune* were freeze dried in 1910. They remained viable for 52.5 years (Ainsworth 1962). The dried state allows them to survive long periods of time, similar to tardigrades and nematodes. In nature this ability could greatly increase the length of the sporing period.

Although there was no decrease in final absorbance of rehydrated *M. oreades* samples, there was an increase in the time it took for samples to begin metabolizing after rehydration. The incubation time required for a visible response increased as the specimens collected in the fall aged. This may have been due to their becoming more fully dry and taking longer to rehydrate.

Since trehalose is common in many species of mushrooms that don't revive, my hypothesis that trehalose content alone was responsible for metabolic or basidocarp revival ability was not confirmed. Trehalose is a storage carbohydrate in fungi. Based on evidence that other anhydrobiotic organsims require trehalose to revive, I believe that trehalose is still a part of the reason

that *Marasmius* spp. revive metabolically.

M. oreades is an excellent example of an anhydrobiotic organism. It shares many characteristics with the better known anhydrobionts. They can be stored for long periods of time and then revived. They can survive periods with no water and when rehydrated they metabolize. In their dry state the samples I microwaved where more resistant to microwaving, longer times were required to kill the enzymatic activity. This was probably due to the fact that microwaves require water to produce heat.

Table 1: A comparison of methanol and ethanol as extraction media for formazan pigment. Absorbance average of 5 samples incubated in TTC for three hours and extracted for two hours in a hot water bath of 55° C.

Table 1: Extraction mediums

Treatment	Avg. Absorb.
Ethanol 95%	0.714
Methanol 100%	0.31

Table 2: Trehalose content of common mushrooms found in a literature search. Trehalose contents are reported in % of dry weight.

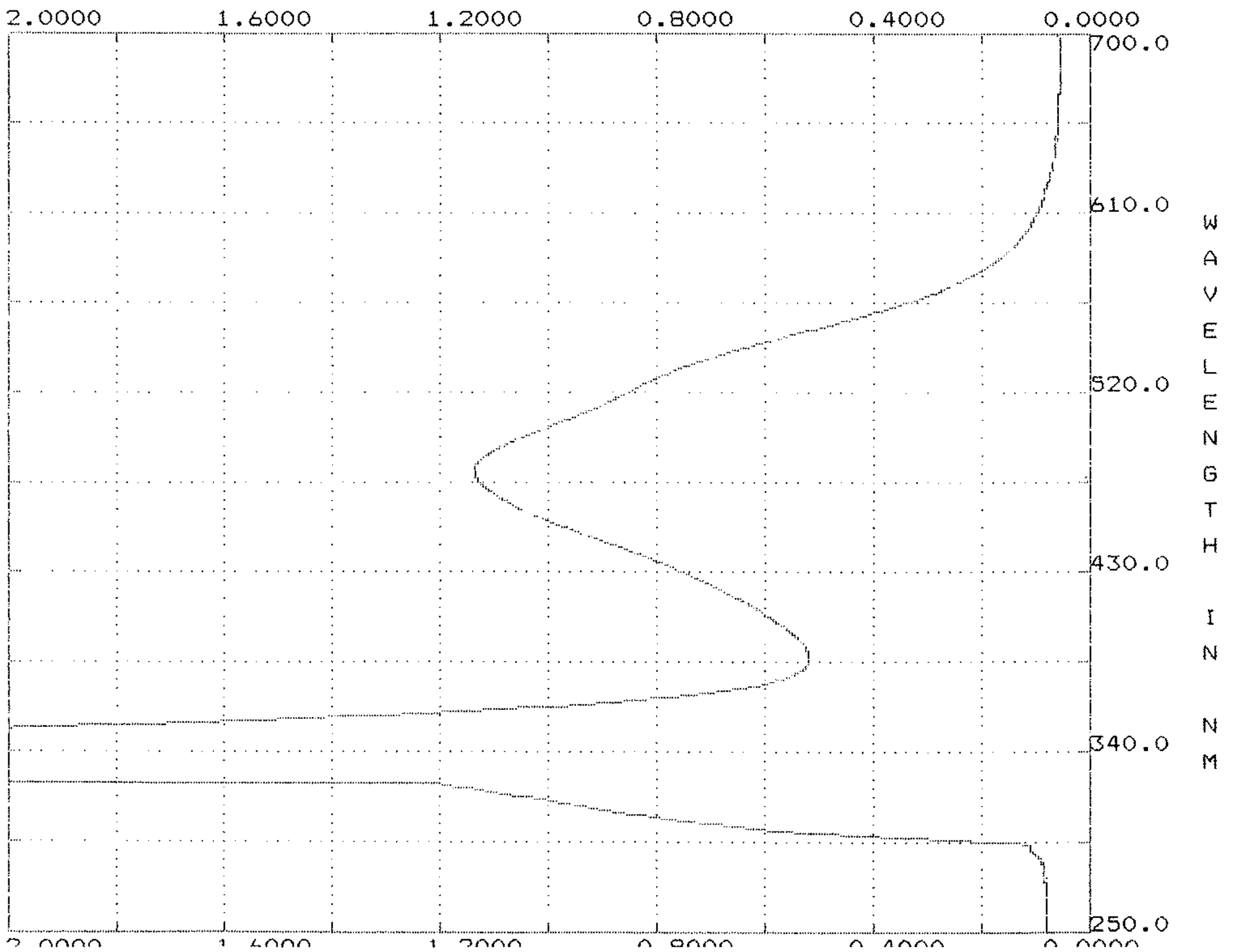
Table 3: A list of *Marasmius* spp that have reviving basidiocarps. Note p.not means that the spp. is unlikely to revive. Three *Collybia* spp that are known to revive are also included.

GENUS	SPECIES	REF.	TREHALOSE	SOURCE
Agaricus	bisporus		0.17%	Br. 1890
Amanita	muscaria	Linn.	0.05%	Br. 1890
Ascobolus	stercorarius	Schroet.	0.00%	Br. 1890
Boletus	appendiculatus	Schaeff.	1.24%	Br. 1892
Boletus	appendiculatus	Schaeff.	0.75%	Br. 1892
Boletus	aurantiacus	Bull.	0.72%	Br. 1890
Boletus	aurantiacus	Bull.	0.00%	Br. 1890
Boletus	badius	Fr.	0.00%	Br. 1892
Boletus	badius	Fries.	0.00%	Br. 1890
Boletus	bovinus	L.	yes	Br. 1890
Boletus	calopus	Fries.	0.00%	Br. 1890
Boletus	chrysenteron	Bull.	0.00%	Br. 1892
Boletus	cyanescens	Bull.	0.00%	Br. 1892
Boletus	edulis	Bull.	2.70%	Br. 1890
Boletus	erythropus	Pers.	0.13%	Br. 1890
Boletus	lanatus	Rostk.	0.00%	Br. 1892
Boletus	luridus	Schaeff.	0.00%	Br. 1890
Boletus	pachypus	Fr.	0.00%	BR. 1892
Boletus	pruinatus	Fr.	0.42%	BR. 1892
Boletus	scaber	Bull.	0.40%	Br. 1890
Boletus	subtomentosus	L.	0.00%	Br. 1890
Boletus	tessellatus	Gill.	yes	Br. 1890
Boletus	veriegatus	Swartz.	yes	Br. 1890
Boletus	veriegatus	Swartz.	yes	Br. 1890
Boletus	versipellis	Fries.	0.41%	Br. 1890
Claviceps	purperea	Tul.	1.02%	Br. 1892
Clitocybe	nebularis	Batsch	yes	Br. 1890
Clitocybe	cyathiformis	Bull.	0.27%	Br. 1890
Clitocybe	nebularis	Batsch.	0.59%	Br. 1890
Collybia	butyracea	Bull.	0.00%	Br. 1890
Collybia	butyracea	Bull.	0.00%	Br. 1890
Collybia	maculata	Al et sch	0.00%	Br. 1890
Collybia	maculata	Al et Schw.	yes	Br. 1890
Coryne	sarcoides	Tul.	0.39%	Br. 1892
Elaphomyces	asperulus	Vittad.	0.00%	Br. 1892
Elaphomyces	echinatus	Vittad.	0.00%	Br. 1892
Elaphomycees	leveillei	Tul.	0.00%	Br. 1892
Elaphonyces	veriegatus	Vittad.	0.00%	Br. 1892
Fistulina	hepatica		0.00%	Br. 1892
Fistulina	hepatica	Huds.	0.00%	Br. 1892
Hygrophorus	agathosmus	Fries.	0.42%	Br. 1892
Hygrophorus	olivaceo–albus	Fries	0.42%	Br. 1892
Hygrophorus	virgieus	Wolf.	traces	Br. 1892
Hypholoma	fasciculare	Huds.	0.00%	Br. 1890
Hypholoma	fasciculare	Huds.	0.41%	Br. 1890
Hypholoma	sublateritium	Fries.	0.42%	Br. 1890
Lactarius	controversus	Bull.	0.00%	Br. 1889 5:1
Lactarius	deliciosus	L.	0.00%	Br. 1889 5:1

GENUS	SPECIES	REF.	TREHALOSE	SOURCE
Lactarius	pallidus	Pers.	0.00%	Br. 1889 5:1
Lactarius	piperatus	SCop.	3.50%	Br. 1889 5:1
Lactarius	piperatus	Scop.	0.00%	Br. 1889 5:1
Lactarius	pyrogalus	Bull.	0.00%	Br. 1889 5:1
Lactarius	rufus	Scop.	0.00%	Br. 1892
Lactarius	subdulcis	Bull.	0.00%	Br. 1889
Lactarius	torminosus	Schaeff.	0.00%	Br. 1889
Lactarius	torminosus	Schaeff.	0.00%	Br. 1889
Lactarius	turpis	Weinm.	0.00%	BR. 1892
Lactarius	vellereus	Fries.	0.00%	Br. 1889
Lactarius	vellereus	Fries.	0.00%	Br. 1889
Lactarius	vellereus	Fries.	0.00%	Br. 1889
Lactarius	vietus	Fries.	0.00%	Br. 1892
Lactarius	blennius	Fries.	0.00%	Br. 1892
Lactarius	quietus	Fries.	traces	Br. 1892
Lactarius	turpis	Weinm	0.00%	Br. 1889
Lactarius	turpis	Weinm	0.00%	Br. 1889
Lactarius	vellereus	Fries	0.00%	Br. 1892
Lactarius	zonarius	Bull.	0.00%	Br. 1892
Lentinus	cochealus	Pers.	1.20%	Br. 1892
Lentinus	tigrinus	Bull.	0.28%	Br. 1892
Marasmius	oreades	Fries	0.35%	Br. 1890
Panus	stipticus	Bull.	0.16%	Br. 1892
Panus	torulosus	Pers.	0.40%	Br. 1892
Paxillus	involutus	Batsch.	0.00%	Br. 1892
Peziza	badia	Pers.	0.00%	Br. 1892
Peziza	onotica	Pers.	0.00%	Br. 1892
Pholiota	adiposa	Fries.	0.20%	Br. 1890
Pholiota	radicosa	Bull.	0.00%	Br. 1890
Pholiota	caperata	Pers.	0.31%	Br. 1890
Pholiota	radicosa	Bull.	0.78%	Br. 1890
Pholiota spe	spectabilis	Fries.	0.69%	Br. 1890
Polyporus	frondosus	Flor.	0.44%	Br. 1892
Poyporus	squamosus	Huds.	0.30%	Br. 1892
Russula	cyanoxantha	Schaeff.	0.00%	Br. 1892
Russula	delica	Vaill.	0.00%	Br. 1892
Russula	faetens	Pers.	0.00%	Br. 1892
Russula	lepida	Fries.	traces	Br. 1892
Russula	ochroleuca	Pers.	0.00%	Br. 1892
Russula	virescens	Schaeff.	traces	Br. 1892
Russula	fellea	Fries.	0.00%	Br. 1892
Tricholoma	flavobrunneum	Fries.	yes	Br. 1890
Tricholoma	pessundatum	Fries.	yes	Br. 1890
Tricholoma	rutilans	Schaeff	0.75%	Br. 1890
volvariella	bombycina	Schaeff.	0.54%	Br. 1890
Xylaria	polymorpha	Pers.	0.00%	Br. 1892
Xylaria	polymorpha	Pers.	0.00%	Br. 1892

GENUS	SPECIES	REF.	REVIVES	SOURCE
Marasmius	alliatus		yes	Gilliam
Marasmius	androsacus	Fries.	yes	Gilliam
Marasmius	armeniacus	Gilliam	p.not	Gilliam
Marasmius	coharens	Cooke&Quelet	yes	Gilliam
Marasmius	borealis	Gilliam	yes	Gilliam
Marasmius	capillaris	Morgan	yes	Gilliam
Marasmius	delectans	Morgan	yes	Gilliam
Marasmius	delectans		yes	Gilliam
Marasmius	elongatipes	Peck	yes	Gilliam
Marasmius	epiphyllus	Fries.	yes	Gilliam
Marasmius	fulvoferrugineus	Gilliam	yes	Gilliam
Marasmius	minutus	Peck	p.not	Gilliam
Marasmius	olidus	Gilliam	yes	Gilliam
Marasmius	oreades		yes	Gilliam
Marasmius	pallidocephalus	Gilliam	yes	Gilliam
Marasmius	pulcherripes	Peck	yes	Gilliam
Marasmius	rotula		yes	Gilliam
Marasmius	siccus	Fries	yes	Gilliam
Marasmius	sullivantii	Montagne	yes	Gilliam
Marasmius	thujinus	Peck	yes	Gilliam
Marasmius	urens		yes	Gilliam
Marasmius	coharens	Cooke&Quelet 12	yes	Gilliam
Collybia	cirrata	Fries.	yes	Gilliam
Collybia	tuberosa	Fries.	yes	Gilliam

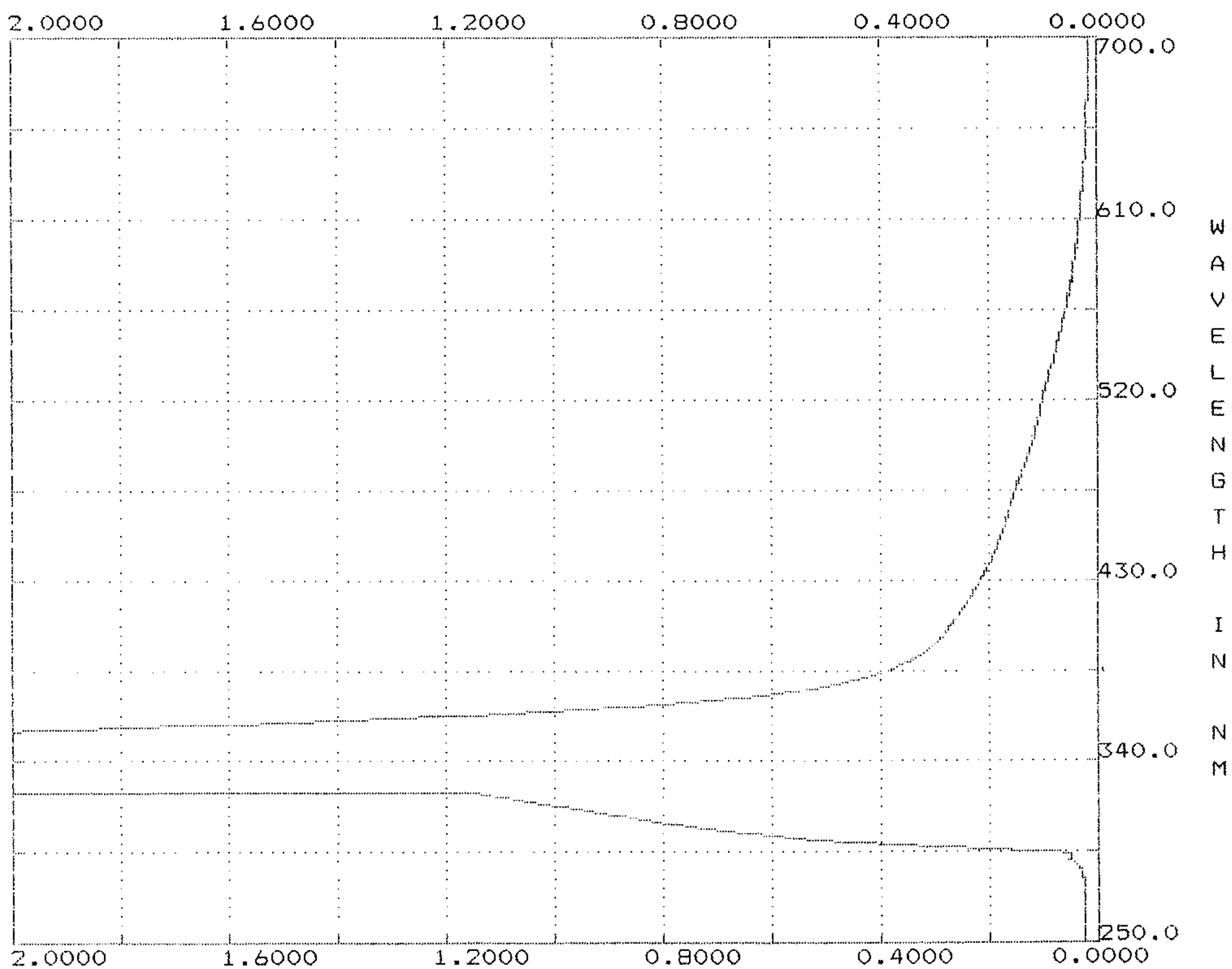
Figure 1: An absorption spectrum of a mushroom sample with high formazan pigment concentration. The sample was incubated for four hours and extracted in methanol for two hours. A 530nm.



BECKMAN
DU-64 SPECTROPHOTOMETER
ABSORBANCE
2.0000
1.6000
1.2000
0.8000
0.4000
0.0000
700.0
610.0
520.0
430.0
340.0
250.0
WAVELENGTH IN NM

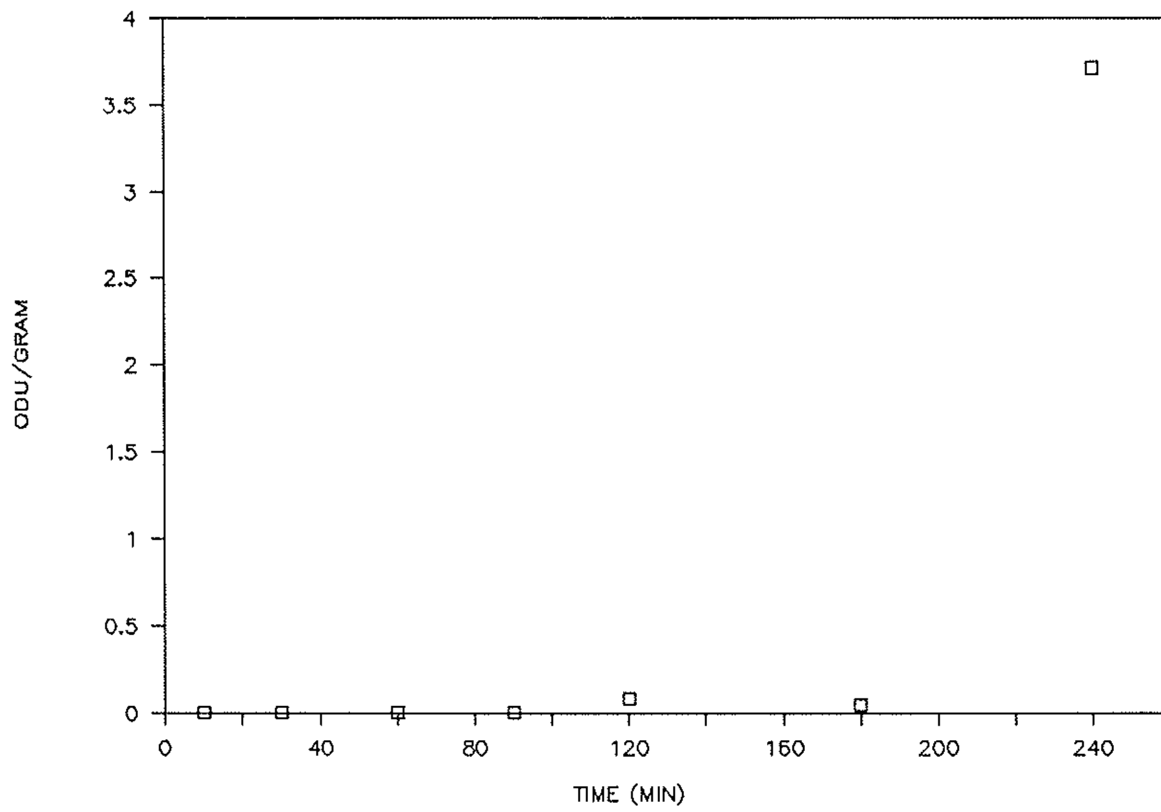
Figure 2: Absorption spectrum of a mushroom sample with formazan pigment absent. All other treatment was kept identical. A 530nm.

Scan Speed: 500 nm/min



Scan Speed: 500 nm/min

Figure 3: Comparison of lengths of time incubated with TTC. The pigment was extracted with 100% methanol for two hours at 35°C. A 530nm.



COMPARISON OF TIMES INCUBATED IN TTC
ODU/GRAM
4
3.5
3
2.5
2
1.5
1
0.5
0
0
40
80
120
160
200
240
TIME (MIN)

Figure 4: Effect of microwaving the samples on high for different lengths of time. Samples were left in the TTC for four hours and extracted in methanol for two hours. A 530nm.

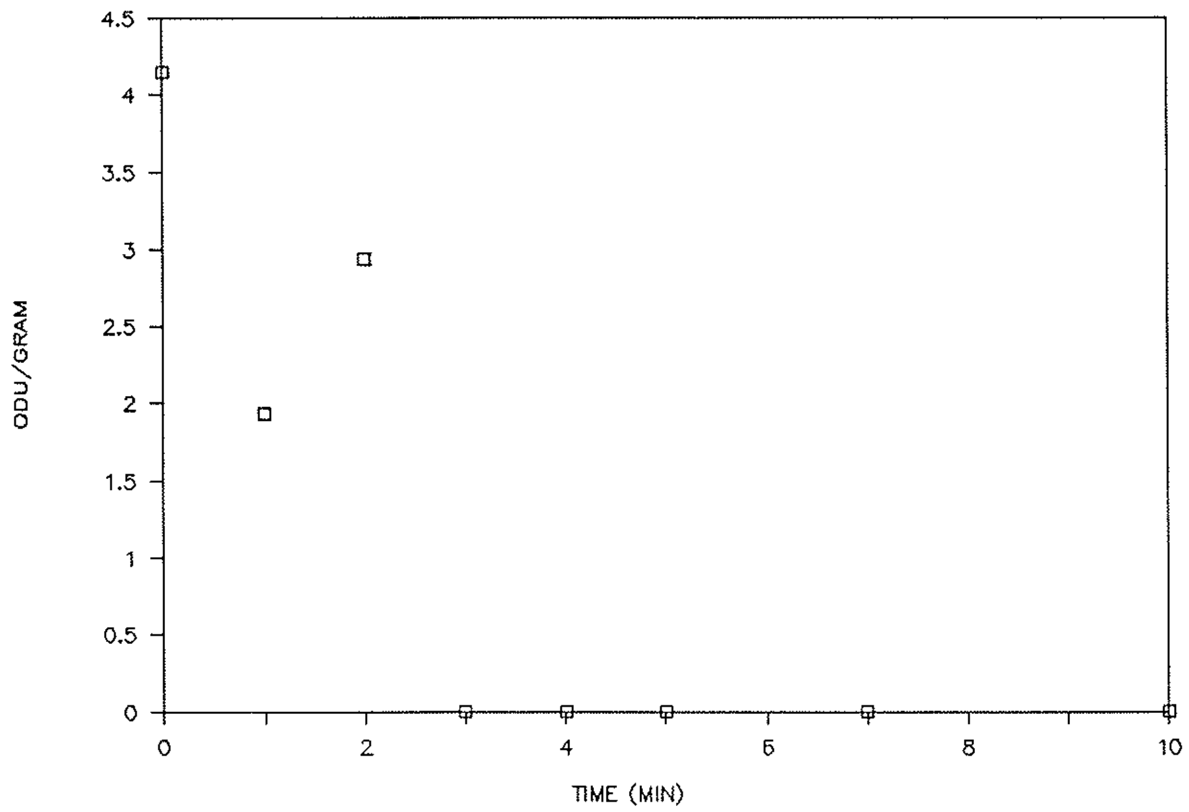
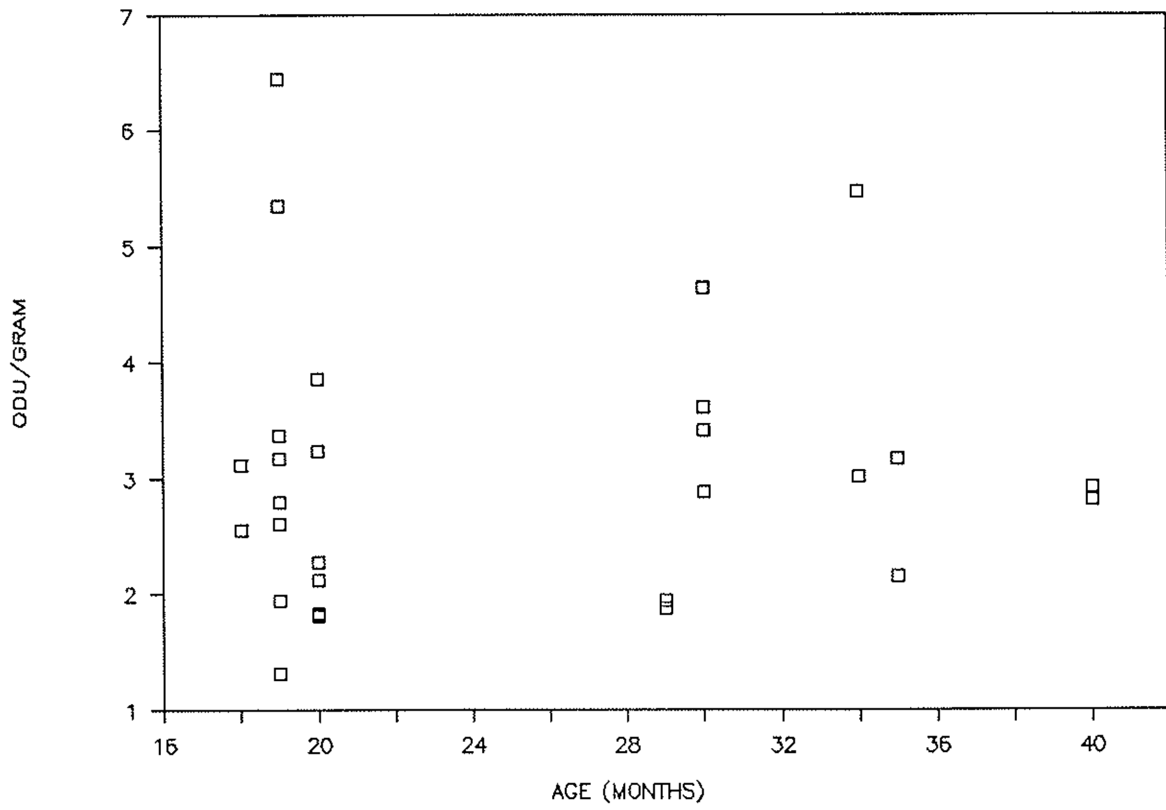

EFFECTS OF MICROWAVING ON REVIVAL
ODU/GRAM
TIME (MIN)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10

Figure 5: Effect of dry storage on *M. oreades* mushrooms. The mushrooms were incubated 10 hours at 35° C and extracted with 100% methanol. A 530nm.



EFFECTS OF AGE ON REVIVAL
ODU/GRAM
AGE (MONTHS)
7
6
5
4
3
2
1
16
20
24
28
32
36
40

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