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*Amanita Muscaria: A Compendium of
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Original Investigations

Monoamines in the Brain under the Influence of Muscimol and Ibotenic Acid, Two Psychoactive Principles of *Amanita Muscaria*

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Abstract. The concentrations of noradrenaline, dopamine and serotonin were measured in the brain of male albino mice and rats after intraperitoneal injections of muscimol, ibotenic acid or LSD. All three drugs induced a generalized increase of serotonin. When muscimol was administered to rats after pretreatment with p-chlorophenylalanine, a serotonin synthesis inhibitor, the serotonin concentration was still increased in midbrain and hypothalamus. Muscimol also caused a reduced accumulation of 5-hydroxyindoleacetic acid in rats pretreated with probenecid. There were differences in the action of the three compounds on the catecholamine concentration. Muscimol and LSD caused a decrease of the catecholamines. Ibotenic acid increased the catecholamine concentration. Certain topographical differences were noted.

The increase in the serotonin concentration in the hypothalamus and midbrain after muscimol may be due to a reduced turnover of serotonin. An increase in serotonin concentration and a decrease of 5-hydroxyindoleacetic acid in the rat brain are effects observed also with other psychotomimetic drugs such as LSD or psilocybin.

Key-Words: Muscimol, Ibotenic Acid, LSD — Monoamine Concentrations in Brain — 5-Hydroxyindoleacetic Acid — p-Chlorophenylalanine — Probenecid — Turnover of Serotonin.

Introduction

Some hallucinogens such as Lysergid (LSD) have been found to cause an increase in serotonin concentration and a decrease in catecholamine concentration in the rat brain (Woolley *et al.*, 1954; Freedman, 1961; Rosecrans *et al.*, 1967).

Two years ago, muscimol, ibotenic acid (Fig. 1) and related compounds were isolated from *amanita muscaria* (Eugster and Takemoto, 1967). Waser (1967) reported some pharmacological and psychotomimetic effects of these two drugs. In mice, their injection produced sympathetic effects which at least in part may be of central origin. The central control

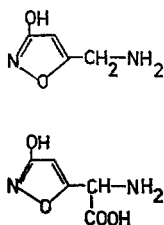


Fig. 1. Molecular structure of muscimol and ibotenic acid. muscimol = 5-amino-methyl-3-hydroxy-isoxazol, ibotenic acid = α -amino-3-hydroxy-5-isoxazol-acetic acid

of motor activity is also impaired with the development in mice of ataxia, catalepsy, convulsions and muscle twitches. With higher doses all treated animals showed sedation.

We investigated the influence of these substances on brain monoamines. Measurements of the monoamine concentrations in the whole brain of mice furnished only general information. More details were obtained by measuring the concentration changes in different regions of the rat brains. In order to gain some insight into the turnover of serotonin, we studied its concentration after administration of a synthesis inhibitor and the concentration of its metabolite, 5-hydroxy-indole-acetic acid (5-HIAA).

Methods

Animals. Male albino mice (20–25 g body weight) of a random bred ICR COBS (cesarian originated barrier sustained) strain from Charles River Mouse Farms, and male albino rats (180–220 g body weight) of a random bred CF (Carworth Farms) strain were used. The animals were provided by the Animal Breeding Institute of the Department of Veterinary Medicine, University of Zürich.

Drugs. The following drugs, dissolved in saline, were administered in total volumes of 0.1 ml/100 g:

muscimol	3 mg/kg i.p.
ibotenic acid	16 mg/kg i.p.
LSD	10 mg/kg i.p.
p-chlorophenylalanine	300 mg/kg i.p.
probenecid	300 mg/kg p.o.
0.9% NaCl	1 ml/kg i.p.
	(control animals)

Brain Sectioning. After decapitation the rat brains were quickly removed and cut just behind the medulla oblongata. The cerebellum,

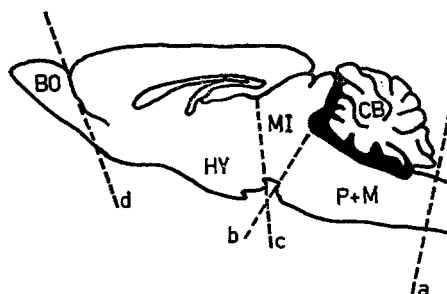


Fig.2. Sagittal section of rat brain (König and Klippel, 1963). The dotted lines (a, b, c, d) represent frontal cuttings. BO bulbus olfactorius; HY hypothalamus with median thalamus and medial hippocampus; MI midbrain; P + M pons and medulla oblongata; CB cerebellum

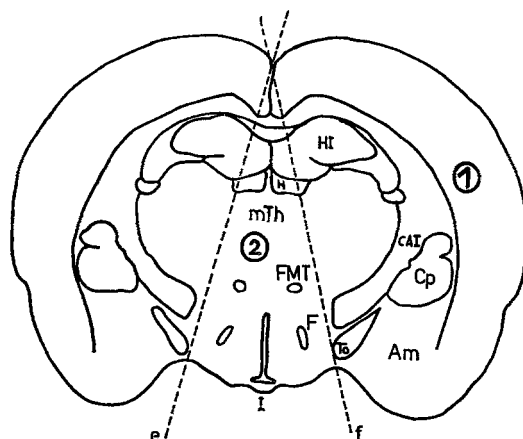


Fig.3. Frontal section of rat brain (König and Klippel, 1963). The dotted lines represent sagittal cuttings (e, f). HI hippocampus; H habenula; mTH median thalamus; FMT fasciculus mamillothalamicus; F columna fornicis; I infundibulum; CAI capsula interna; Cp nucleus caudatus putamen; Am amygdala; To tractus opticus. 1. "Forebrain" contains cortex, striatum, amygdala and lateral parts of thalamus. 2. "Hypothalamus" contains hypothalamus, median thalamus and medial hippocampus

the olfactory bulb and the pineal gland were discarded. Amine determinations were performed in four brain areas: Medulla oblongata + pons, midbrain, hypothalamus + median thalamus, remaining forebrain. The position of the various cuts is given in Figs.2 and 3.

Fluorimetric Determination. Noradrenaline was determined according to Bertler *et al.* (1958), dopamine according to Carlsson and Waldeck (1958) and 5-hydroxytryptamine according to Udenfriend *et al.* (1958).

Table 1. *Monoamine concentrations (ng/g fresh tissue) in the mouse brain two hours after the i.p. injection of 16 mg/kg ibotenic acid, 3 mg/kg muscimol or 10 mg/kg LSD*

		Ibotenic acid	Muscimol	LSD
		$\pm$ S.D. (n)	$\pm$ S.D. (n)	$\pm$ S.D. (n)
NA	T	539 $\pm$ 80 (5)	447 $\pm$ 44 (3)	249 $\pm$ 76 (5)
	C	485 $\pm$ 55 (3)	474 $\pm$ 47 (3)	269 $\pm$ 76 (7)
DA	T	426 $\pm$ 30 (4)	432 $\pm$ 51 (3)	519 $\pm$ 172 (7)
	C	354 $\pm$ 37 (4)	367 $\pm$ 34 (3)	603 $\pm$ 100 (7)
		$p \leq 0,0025$	$p \leq 0.05$	
5-HT	T	363 $\pm$ 77 (11)	399 $\pm$ 83 (8)	342 $\pm$ 15 (3)
	C	368 $\pm$ 81 (8)	360 $\pm$ 91 (6)	300 $\pm$ 25 (3)
			$p \leq 0.10$	$p \leq 0.05$

Abbreviations for Table 1—4.

<i>S.D.</i>	standard deviation
(<i>n</i>)	number of experiments each with 4—5 animals
<i>T</i>	mean concentration in the test brain tissue
<i>C</i>	mean concentration in the control brain tissue
<i>p</i>	confidence coefficient
<i>FB</i>	forebrain
<i>HY</i>	hypothalamus
<i>MI</i>	midbrain
<i>P + M</i>	pons and medulla oblongata.

with slight modifications (Häggendal, 1962). We added 0.1 ml of 2% ascorbic acid and 0.2 ml of 10% EDTA to 10 ml aqueous solution. 5-HIAA was estimated fluorimetrically as described by Roos (1962) and Sharman and Smith (1962). In the experiments on mice, 4 whole brains were pooled, whereas in the studies on rats 5 brain sections were used for one determination. Care was taken to start the experiments always at the same time of the day. The average recovery of the monoamines which were added to tissue homogenate and carried through the procedures ranged between 85 and 95 percent. The values in the tables are corrected for this recovery.

Statistical Analysis. Differences of the fluorescence intensity between test and control samples were analysed by means of the *F*-test and *t*-test comparison (Doerffel, 1965). Differences between the means of treated and control animals are considered to be significant if the confidence coefficient is 95% ($p < 0.05$) or more.

Results

There is some similarity between the effects of muscimol and LSD on the overall monoamine concentration in the mouse brain (Table 1).

Table 2. *Monoamine concentrations in four different regions of rat brain (ng/g fresh tissue) two hours after the injection of ibotenic acid (16 mg/kg i.p.)*

		<i>FB</i>	<i>HY</i>	<i>MI</i>	<i>P + M</i>
		$\pm$ S.D. (<i>n</i>)	$\pm$ S.D. (<i>n</i>)	$\pm$ S.D. (<i>n</i>)	$\pm$ S.D. (<i>n</i>)
NA	<i>T</i>	195 $\pm$ 84 (4)	752 $\pm$ 306 (4)	1056 $\pm$ 428 (4)	513 $\pm$ 179 (4)
	<i>C</i>	229 $\pm$ 102 (8)	531 $\pm$ 237 (6)	542 $\pm$ 161 (7)	404 $\pm$ 110 (6)
		$p \leq 0.10$		$p \leq 0.01$	
DA	<i>T</i>	366 $\pm$ 77 (4)	768 $\pm$ 209 (4)	465 $\pm$ 43 (4)	390 $\pm$ 245 (4)
	<i>C</i>	384 $\pm$ 86 (5)	574 $\pm$ 146 (5)	398 $\pm$ 141 (6)	177 $\pm$ 30 (5)
			$p \leq 0.10$		$p \leq 0.05$
5-HT	<i>T</i>	294 $\pm$ 68 (5)	721 $\pm$ 82 (5)	804 $\pm$ 47 (4)	454 $\pm$ 87 (5)
	<i>C</i>	259 $\pm$ 70 (7)	612 $\pm$ 117 (9)	685 $\pm$ 101 (9)	413 $\pm$ 83 (9)
			$p \leq 0.05$	$p \leq 0.025$	$p \leq 0.10$

Table 3. *Monoamine concentrations in four different regions of rat brain (ng/g fresh tissue) two hours after the injection of muscimol (3 mg/kg i.p.)*

		<i>FB</i>	<i>HY</i>	<i>MI</i>	<i>P + M</i>
		$\pm$ S.D. (<i>n</i>)	$\pm$ S.D. (<i>n</i>)	$\pm$ S.D. (<i>n</i>)	$\pm$ S.D. (<i>n</i>)
NA	<i>T</i>	200 $\pm$ 49 (5)	645 $\pm$ 250 (5)	392 $\pm$ 205 (5)	389 $\pm$ 122 (5)
	<i>C</i>	241 $\pm$ 44 (4)	765 $\pm$ 315 (5)	430 $\pm$ 81 (5)	554 $\pm$ 103 (5)
					$p \leq 0.05$
DA	<i>T</i>	548 $\pm$ 209 (5)	961 $\pm$ 234 (4)	552 $\pm$ 389 (5)	393 $\pm$ 104 (4)
	<i>C</i>	809 $\pm$ 179 (5)	1245 $\pm$ 339 (4)	772 $\pm$ 334 (4)	326 $\pm$ 106 (4)
		$p \leq 0.05$			
5-HT	<i>T</i>	356 $\pm$ 85 (5)	819 $\pm$ 321 (4)	808 $\pm$ 128(5)	560 $\pm$ 196 (5)
	<i>C</i>	275 $\pm$ 43 (6)	670 $\pm$ 248 (4)	706 $\pm$ 119 (5)	464 $\pm$ 139 (5)
		$p \leq 0.05$			

The serotonin concentration increased and the noradrenaline concentration decreased. However the dopamine concentration increased after muscimol in contrast to a decrease after LSD.

After ibotenic acid an increase was observed in noradrenaline and dopamine concentrations in the mouse brain, whereas the serotonin concentration did not change. The relatively small changes in monoamine concentration in the mouse brain could originate from large changes in small brain areas. Therefore, the influence of muscimol, ibotenic acid and LSD was investigated in four different regions of the rat brain (Tables 2 and 3).

Following the injection of ibotenic acid the noradrenaline content was markedly increased in the hypothalamus, midbrain and pons

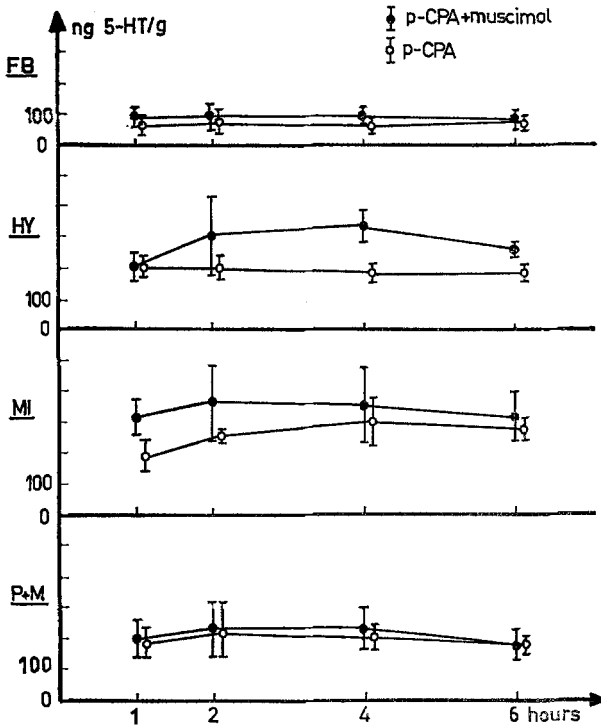


Fig. 4. Serotonin concentration in rat brain one, two, four and six hours after injection of muscimol (3 mg/kg i.p.). The animals were pretreated with p-chlorophenylalanine (300 mg/kg i.p.) 48 hours before the muscimol injection. *FB* forebrain; *MI* midbrain; *HY* hypothalamus; *P + M* pons and medulla oblongata

+ medulla oblongata (Table 2). The dopamine concentration was increased in the hypothalamus and pons + medulla oblongata, and showed a tendency to rise in the midbrain and to fall in the forebrain. Serotonin concentrations increased especially in hypothalamus and midbrain.

After the injection of muscimol the noradrenaline concentration was markedly lowered in pons and medulla oblongata (Table 3), and a similar tendency was found in all other regions. Dopamine decreased markedly in the forebrain. A slight decrease was also observed in the hypothalamus and midbrain, with an increase in pons and medulla oblongata. Serotonin levels were distinctly increased in all parts, but the change was only significant in the forebrain.

The generalized increase of serotonin induced by muscimol, ibotenic acid as well as by LSD suggested further investigations. 48 hours after administration of p-chlorophenylalanine, which inhibits the 5-HT syn-

Table 4. 5-hydroxy-indole-acetic acid concentration (ng/g fresh tissue) in four different regions of rat brain one hour after the injection of muscimol (3 mg/kg i.p.). The animals were pretreated with probenecid (300 mg/kg p.o.) for two hours

	FB	HY	MI	P + M
	$\pm$ S.D. (n)	$\pm$ S.D. (n)	$\pm$ S.D. (n)	$\pm$ S.D.(n)
5-HIAA T	586 $\pm$ 227 (5)	677 $\pm$ 128 (6)	1305 $\pm$ 222 (6)	857 $\pm$ 91 (6)
C	601 $\pm$ 220 (6)	931 $\pm$ 216 (5)	1625 $\pm$ 377 (6)	1283 $\pm$ 364 (5)
		$p \leq 0.025$	$p \leq 0.05$	$p \leq 0.01$

thesis by blocking the tryptophan hydroxylase, the serotonin concentration was markedly reduced to 20% of the normal value in all brain regions (Fig.4). The value of the control group showed slight changes which could be due to diurnal variations. After this pretreatment, muscimol increased the serotonin concentration in the midbrain and hypothalamus. The effect of muscimol was noticed first in the midbrain and lasted 6 hours in the hypothalamus (Fig.4).

The measurement of 5-HIAA, the acid metabolite of 5-HT, was expected to yield information about the turnover of 5-HT. Pretreatment with probenecid blocks the permeation of 5-HIAA through the blood-brain-barrier (Neff and Tozer, 1968) and thus amplifies certain differences in the 5-HIAA concentration. Two hours after pretreatment with probenecid there was a significant decrease of 5-HIAA induced by muscimol (Table 4).

Discussion

All three drugs investigated increased the serotonin concentration in the brain, the effects differing only quantitatively from each other. The findings after LSD are in agreement with earlier investigations (Daniel and Freedman, 1963; Rosecrans *et al.*, 1967).

When the synthesis of 5-HT was blocked by p-chlorophenylalanine, muscimol still increased the serotonin concentration in hypothalamus and midbrain, but it lowered the formation of 5-HIAA. The increase in serotonin concentration brought about by muscimol may be due in part to a decreased release and/or metabolism of 5-HT. Inhibition of monoamine oxidase is unlikely considering the observed symptoms (sedation and hypothermic effects) and the decrease in catecholamine concentrations. The decreased turnover rate could rather result from a diminished 5-HT release by neurons due to a decrease of the impulse flow in the serotonergic neurons (Andén *et al.*, 1968; Corrodi and Malmfors, 1966).

5-HT concentration was affected by muscimol and ibotenic acid to about the same degree in all brain regions. After inhibition of 5-HT syn-

thesis forebrain, pons and medulla no longer showed an increase of 5-HT after muscimol. The elevated hypothalamic concentration, when considered together with the low 5-HIAA formation, suggested a reduced release of 5-HT. Besides this, a change in the rate of synthesis might have played a role in the increase in 5-HT concentration observed without synthesis inhibition. It is interesting to note that after synthesis inhibition muscimol produced initially a rise of 5-HT concentration in the midbrain which contains the bulk of the nerve cell bodies (Dahlström and Fuxe, 1964), and this was later reduced to control values. At the time of maximal sedation (2–4 hours after injection of muscimol) the change was more pronounced in the nerve terminal region.

There are certain differences between the action of the three compounds investigated on the catecholamine concentrations: muscimol and LSD were similar in their effect on the monoamine concentration in the rat brain, both inducing a decrease in catecholamines and an increase in serotonin. The same is true for noradrenaline and serotonin in the mouse brain, whereas in this species the two drugs showed different effects on the dopamine concentration. The reason for the difference between the increase in dopamine concentration after muscimol in the mouse brain and the decrease in the rat brain is unknown.

In contrast to these two drugs ibotenic acid increased the catecholamine concentration. Certain topographical differences in the magnitude of the change in NA and DA concentrations were noted (Tables 2 and 3), but they were rather irregular and do not permit many conclusions to be drawn on the topography of CA-containing systems. The fact that after ibotenic acid dopamine concentration remained practically unchanged in the forebrain (with basal ganglia) and in the midbrain, might indicate that its concentration in the nigro-neostriatal system did not alter and thus, that the changes observed especially in pons and medulla might result from its occurrence as a precursor of NA. In contrast, muscimol markedly reduced the DA concentration in the forebrain and the midbrain, which suggests that besides NA neurons it also affected DA neurons. The significance of the changes in the catecholamine concentrations with respect to turnover changes has not yet been studied.

It does not seem possible at the present time to establish a close correlation between the functional effects of these drugs and their biochemical actions. Substances with LSD-like behavioral effects, such as psilocybin, mescaline or 1-acetyl-LSD produce an increase in brain 5-HT concentration and a decrease in catecholamine concentration (Freedman, 1963; Giarmann and Freedman, 1965; Aghajanian and Freedman, 1967). Rosencrans *et al.* (1967) found that after the injection of LSD (200 µg/kg i.v.) into rats the 5-HT concentration increased and at the same time the

5-HIAA concentration decreased in the rat brain. This could be due to a diminished release of 5-HT.

Since muscimol and, to a lesser extent, ibotenic acid possess certain psychotomimetic properties (Waser, 1967; Theobald *et al.*, 1968), it is tempting to look for correlations between such symptoms and certain similarities in the biochemical effects, notably that on serotonin. One common action of all three drugs was possibly to lower the impulse flow in the serotonergic neurons. It remains to be elucidated whether this was due to a stimulation of serotonin receptors with consequent negative feedback action on the respective neurons, as suggested by Andén *et al.* (1967, 1968) and Corrodi *et al.* (1967), or to a transsynaptically induced reduction of activity in these neurons.

However, the biochemical effects need not be correlated exclusively to behavioral changes. They may also reflect an involvement of neurons of other functional systems, e.g. central representations of vegetative functions. With regard to the changes in DA concentration in the mouse brain, the similarity of the actions of both muscimol and ibotenic acid on the dopamine concentration in this species, and the fact that both drugs caused extrapyramidal symptoms is most interesting.

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AMANITA MUSCARIA AND THE THUNDERBOLT LEGEND IN GUATEMALA AND MEXICO

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Early man was profoundly moved by the apparently miraculous attributes of lightning and thunder which first inspired in him fear, then respect and reverence for the power his gods displayed in those awesome phenomena. Of particular concern to the ethnomycologist is the causal association in the mind of primitive man of thunderbolts and mushrooms and among the cultures in which this magical alliance became integrated into national mythologies were the Roman and the Hindu. In Roman lore, some fungi were believed to spring from the ground in places struck by a thunderbolt and according to Hindu tradition as recorded in the Rig Veda, it is stated that "Parjanya, the god of thunder was the father of Soma" (Wasson, 1969). Wasson minutely examined the available evidence for the identification of soma and concluded that the most convincing interpretation we may hope for at present is its identity with *Amanita muscaria* (L. ex Fr.) Hooker.

During the past several years, I have examined some aspects of ethnomycology in the Americas, principally those relating to mushroom stones and Maya codices (Lowy 1968, 1971, 1972a, b). As a result of a study made in Guatemala, Mexico, and Honduras in June and July, 1973, it was established that there exists in the Guatemalan highlands and in southern Mexico a tradition that links *Amanita muscaria* with the thunderbolt. Preliminary inquiries in western Honduras have thus far failed to reveal a trace of this legend, nor have I found it in El Salvador. The first intimation that such a mythology might be extant among indigenous peoples of the Americas came in 1970 in the course of my field work in Mexico and Guatemala. Although I had by that time compiled a small mushroom vocabulary in several Indian languages, further opportunities were sought for the verification of the meanings of some words from other native informants and through consultation with professional linguists. Sufficient evidence is now at hand to justify the assertion that in some regions of the New World the *Amanita muscaria*-thunderbolt legend is firmly implanted in the minds of its autochthonous inhabitants.

While I was interrogating three young bilingual (Quiche-Spanish speaking) Indians who were participants in the linguistic "Proyecto Francisco Marroquín" in Antigua, Guatemala in June, 1973, these in-

formants unequivocally identified *A. muscaria* from a color photograph. Their name for this mushroom in the Quiche idiom was “kaquljá:.” When asked what the word meant, they translated it into Spanish as “trueno” meaning thunder. After consultation among themselves and questioning by myself and Dr. William Norman, Quiche specialist, it was evident that it was not thunder alone that was signified by “kaquljá:,” but the accompanying lightning as it strikes the ground during a storm. The equivalent term in Spanish is “rayo.” This was clearly differentiated from the Quiche word “xkoyopá:,” or “relámpago” in Spanish, a lightning flash, which, according to my informants, was never used to designate the mushroom. It is of interest too, that the native speakers could offer no explanation for the use of the word “kaquljá:” for *A. muscaria*, indicating only that it was common usage. Its ancestral origins have long since been forgotten. To my knowledge, this is the first report that the thunderbolt and *Amanita muscaria* have been found to be intimately related in the folklore of an indigenous population in the Americas.

At the “Proyecto,” *Amanita caesarea* (Scop. ex Fr.) Pers. ex Schw. was easily recognized from my color photograph by the native speakers of Quiche, Cakchiquel, and Mam. It was called “q’atzu:” (or by one of its variants “atzui” or “atzu”) by the Quiche, a word which I had confirmed on many previous occasions in the Quiche-speaking regions of Guatemala. Among the Cakchiquel of Sololá and Panajachel, *A. caesarea* is known as “q’uatzu:y” which was verified by Dr. Norman’s Cakchiquel group at the “Proyecto.” Lic. Nora England, specialist in the Mam language there, kindly allowed me to question her native Mam students and from them the word “xq’antzy:” was given for *A. caesarea*, but a photograph of *A. muscaria* was not recognized by them. The Cakchiquel group also identified *A. muscaria* from the photograph, knew it to be poisonous and called it “ruk’awach q’uatzu:y,” the poisonous “q’uatzu:y,” but no other connotation was associated by them with this mushroom.

A few kilometers north of Chichicastenango, in pine woods along the road to El Quiche, I again collected (July, 1973) both *A. muscaria* and *A. caesarea* and returned with the specimens to the Sunday market at Chichicastenango. There I sought the mushroom venders and displayed my finds. The edible and poisonous species were immediately distinguished and I was strongly admonished not to eat the “kaquljá:” for it would have fatal consequences. Whereas “kaquljá:” refers to the legendary origin of *A. muscaria*, a Quiche expression that is descriptive of its dangerous, fearful potentialities is “itzel ocox,” meaning the evil or diabolical mushroom.

In Comitán, San Cristóbal de Las Casas, and Tuxtla Gutiérrez in the state of Chiapas, Mexico, *A. caesarea* is a common and highly prized mushroom called “yuyo” (or “yullo”) which in some regions of Latin America is a term used to signify “hierbas tiernas comestibles” (in “Diccionario de la lengua Española”, Madrid, 1970), tender edible herbs. It is frequently prepared by roasting over a charcoal fire and I have eaten it in public places in San Cristóbal on numerous occasions. Incidentally, I have consistently found in my travels throughout the Americas that among lay individuals with a formal education, varying degrees of mycophobia is the general rule, but the educationally less privileged tend to be distinctly mycophilic. This is not as paradoxical as it may appear, since the peasantry are closest to the soil and know at first hand its products both cultivated and wild. *Amanita muscaria* is well known as a poisonous mushroom throughout the region of Chiapas under discussion and the revealing Spanish epithet for it in San Cristóbal de Las Casas and in Tuxtla Gutiérrez is “yuyo de rayo,” the “yuyo” of the thunderbolt. I also discovered that among the Tzeltal-speaking natives of San Cristóbal and nearby Zinacantán, this expression has its exact counterpart in their language. *Amanita muscaria* is called by them “yuy chawk,” “yuy” being a variant of the Spanish “yuyo” and “chawk” meaning “rayo” or thunderbolt.

Chiapas and Guatemala are not only geographically contiguous, but, more significantly, they share a common heritage, so it may be surmised that the similar mushroom mythologies that find expression there reflect the influence of a dominant Mayan civilization. It may only be conjectured how this legend came to be shared by such widely separated cultures as are those of the New World and the Indo-European, but it is not improbable that it arose independently among the Maya. Further inquiries among contemporary Guatemalan linguistic groups including the Cakchiquel, Kekchi, Mam, and Ixil in Guatemala and the Tzotzil, Tzeltal, Chontal, and others in Mexico are planned so that it may eventually be possible to trace the range and depth of this ancient tradition in the Americas.

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***FUSICOCCUM ELAEAGNI*, THE CAUSE OF A CANKER AND
DIEBACK OF RUSSIAN OLIVE, REDESCRIBED AND
REDISPOSED TO THE GENUS *PHOMOPSIS*¹**

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Carter and Sacamano (1967) described *Fusicoccum elaeagni* and reported its pathogenicity on *Elaeagnus angustifolia* L. (Russian olive) in Missouri. In a subsequent note, Carter and Dodd (1969) reported the discovery and distribution of *F. elaeagni* in Illinois. This species was first detected in Canada on nursery stock in 1968. It was subsequently found in Canada on *E. angustifolia* nursery stock, especially on seedlings recently imported from Europe, in 1969 and 1970 when a survey for this fungus was carried out by officers of the Canadian Plant Protection Division (Arnold and Straby, 1973).

In single-conidial cultures isolated from a culture made from the type specimen of *F. elaeagni* (ILLS 34453) and grown on 2% potato dextrose agar (PDA), pycnidial stromata typical of the form genus *Phomopsis* were formed. They contained both the α -conidia previously described by Carter and Sacamano, and β -conidia. Both types of conidia were found also in pycnidial stromata formed on the roots of one collection of diseased *Elaeagnus angustifolia* seedlings made in British Columbia, Canada. On the basis of this new information, this species, *Fusicoccum elaeagni* Carter & Sacamano, is transferred to the form genus *Phomopsis* to which it belongs.

¹ Plant Research Institute Contribution No. 975.

sites are enclosure units (basically hut and livestock enclosures), terraces used for agricultural purposes, and stone-walled tracks between the terraces from the outside of the settlement to the enclosure units. Sites are all on sloping ground on the sides of valleys. While a number of these sites were visited, particular attention was paid to a settlement on the farm Badfontein. This is a large village site covering ca. 3 sq. mi. on the banks of the Crocodile River, approximately 17 mi. south of Lydenburg. Two enclosure units, 44/71 and 45/71, were excavated, and both provided information about household arrangements, material culture, and probable cultural affinities.

Pottery resembles both the material recovered by Laidler and modern Pedi pottery. The principal type is a short-necked pot with a row of punctuation marks just below the rim, a large chevron or arcade motif on the shoulder, and differential colour burnishing, usually graphite and red ochre. Shoulder decoration and burnishing do not always occur. A number of pots have strong affinities with some from the Eastern Transvaal lowveld.

Evidence for agriculture is seen in the terracing and in the finding of a hoe, much worn down by sharpening.

Many of the stones among the terraces have evidence for use as sharpening stones for metal tools. Pastoralism is shown by bones of cattle and sheep/goats, as well as the tracks and cattle kraals.

Associated with some settlements are refuge sites, usually caves (as at Sudwala and Ngodwana) but in two cases isolated cliff-bound heights (Mapochstad [Mason 1962] and Marapan's Rock). Refuge sites and the presence of trade beads of recent type suggest a date in the late 18th and 19th centuries, when Ndebele and Swazi invaded the area, shortly followed by the first European settlers. Refuge sites were used as late as the 1870s by Pedi people during the Sekhukhune wars (Aylward 1881).

Evidence for metal working is lacking at Badfontein, though slag has been found at a site in the Lydenburg Municipal grounds, scheduled for excavation in the near future.

The sites show strong cultural affinities with modern Pedi settlements. One of the sites excavated at Badfontein has been compared with the layout of a modern Pedi *kgoro* (Mönnig 1967), and the resemblance is striking. That, with the strong resemblances in settlement layout and pottery and the fact that in the late 18th and 19th centuries

the Pedi are known to have had an extensive empire that included the Lydenburg region (Hunt 1931), suggests conclusively that late Iron Age settlement in the escarpment area of the Eastern Transvaal can be attributed to Pedi people.

A study of the Iron Age of Inyanga, Rhodesia, shows the only point of comparison between Inyanga and the Eastern Transvaal to be the layout of settlements with terraces, tracks, and enclosure units. Details of the enclosure units and other features of the material culture are very different in the two areas.

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Russian Use of *Amanita muscaria*: A Footnote to Wasson's *Soma*¹

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In *Soma*, a book which has attracted worldwide attention, Wasson (1968) documents his conviction that *Soma*, the god and the plant celebrated in the Vedic hymns, was a mushroom, *Amanita muscaria*, or fly agaric. Furthermore, he shows that this red-capped, white-spotted mushroom had and continues to have an important place in the folklore and folk life of a number of peoples, from the small peoples of the Soviet North to the Slavs. In March 1971, Wasson wrote to me, asking what I knew of the present use of *A. muscaria* among the small peoples of the Soviet North. My husband and I have done considerable research on culture change among the small peoples, and, in addition, we translated Okladnikov's *Yakutia* (1970). Our only reference to *A. mu-*

scaria at that time was a statement (Gurvich and Kuzakov 1960:171, translation mine) that "some Koryaks—representatives of the older generation—collect *Amanita muscaria* mushrooms, dry them, and then use them as narcotics." I agreed to do some research on the topic for Wasson, because I wanted very much to know whether the Russians ever used *A. muscaria* ritually. Siberia has been settled by Russians since the mid-17th century. Russian religious dissidents made up a significant percentage of these settlers. According to the Russian literature, these sectarians indulged in a number of practices designed to facilitate the descent of the Holy Spirit. Such descriptions as I have read (and in the great majority they have been written by hostile observers) seem to be describing a basically shamanistic activity. Would such people, in the normal course of events, have employed *A. muscaria*?

Although every conceivable charge has been levelled against Russian sectarians, the use of *A. muscaria* has never

been one of them. On the other hand, Russian and Soviet sources admit that Russians in Siberia do make use of native shamans and that there have been Russian shamans (Borgoraz 1899:120; Manzhigeev 1962; Vorob'ev 1926). Furthermore, the Soviet commentary on Wasson's work (Elizarenkova and Toporov 1970; Dikov 1971:24-26) clearly indicates that the ritual use of *A. muscaria* by any given people is viewed as an indication of the antiquity, nature, and extent of shamanism over a wide area. The exchange of letters between Wasson and myself for more than a year suggests that he has a lively interest in certain aspects of folklore (snakes, one-legged and one-eyed beings or gods, and world trees) which will eventually broaden and deepen the work sketched in *Soma* and in *Mushrooms, Russia and History* (Wasson and Wasson 1957).²

²I have discovered that the Nivkhi (Gilyaks) once wore a charm to prevent or cure illness which was armless and legless, essentially a head with a long smooth body. This charm, when worn on the breast, was called *panghk* (Shrenk 1903:110; pl. LIII, LIV show somewhat similar idols among the Golyd [Nanai]). Wasson connects this

¹This report is an expanded version of a paper presented to the Kroeber Anthropological Society, Berkeley, Calif., on May 21, 1972.

It very quickly became apparent that among the Russians, the use of *A. muscaria* was twofold: as a medicinal ingredient, and as a substitute for alcohol. Since comparatively little has appeared in English on this topic, I will describe these uses in some detail, taking the substitute for alcohol first.

The use of *A. muscaria* in the village of Markovo, in the Anadyr region of the Chukot Peninsula, was described by Baron Gerhard von Maydell (cited in Wasson 1968:254–55) in the 1860s. A more detailed description is contained in a manuscript by D'iachkov (1893:114–18), a resident of Markovo, who was perhaps at a disadvantage because he was simply an observer and not a participant. Nevertheless, the report is of some interest: The local inhabitants, when looking for *A. muscaria*, believed that if they ate the first one they found they would inexorably be led toward others. If a person took the *A. muscaria* alone, his visions were more terrible than if he consumed it in company. The smallest dose was three; more could be added to intoxication, but not everyone was affected in the same degree. The mushrooms were generally eaten whole, but were sometimes mixed with other food, or boiled in water, which was then drunk. The practice was to sit in a circle eating three or more mushrooms, after which all the participants returned to their houses. However, in some manner, they remained in communication with each other, to the extent that they knew who was intoxicated and who wasn't. Apparently the purpose of getting to-

gether at all was to have one person masticate the mushroom and then pass it on to the next, with whispered suggestions about what the person would see. D'iachkov said that a person who knew nothing about shamanism would begin to act like a shaman as soon as he ate the *A. muscaria* and that shamans who were asked to heal the sick or "divine some secret matter" often asked for *A. muscaria* beforehand. It is perhaps significant that D'iachkov did not mention religious motivation, although he reported that the intoxicated person said that he had seen heaven and hell, or paradise. We should remember that the 19th-century Russian peasant was far from secularized, and that heaven, paradise, and hell were very much part of his intellectual frame of reference.

When D'iachkov's manuscript was published, his first name and history were unknown, but he was said to have been a Russified Chuvanets. The question arises, therefore, whether D'iachkov's observations relate to Russians at all. It seems that inadvertently I stumbled on a very important problem of Russian and Soviet nationality policy—namely, at what point can a small ethnic troupe be considered Russian if some element of it at one time was non-Russian? Scattered references in *Peoples of Siberia* (Levin and Potapov 1964) alternately list the Chuvantsy as one of the small peoples of the North and place them in a description of Russians in Siberia (p. 113, where they are described as one of the Yukagir tribes, and also pp. 491, 506, 788, 790,

800, 801; but on p. 820 we read that the settled Chuvantsy may now be considered Russians, whereas the reindeer Chuvantsy have merged with the Chukchi; on p. 830, Chuvantsy are coupled with "local Russians"). A sketch in *Izvestiia* (June 15, 1972) by the journalist A. Pushkar' adds a few details to D'iachkov's rather hazy description. D'iachkov was a psalm-reader and the first teacher in Markovo; his first name was Afanasii. Markovo was a trading center in the late 18th century, and the inhabitants were Russified Chuvantsy who had taken the surnames, language, customs, and songs of the Cossacks who had originally come into the area with Semen Dezhnev. D'iachkov himself knew only a few words of the Chuvan language, having picked them up from his grandmother, who was by no means fluent. On a number of counts, therefore, we can say that the inhabitants of Markovo are Russian now and were when D'iachkov wrote. S. A. Tokarev (personal communication, June 25, 1972) confirms this supposition, writing "this is a small isolated group of Russian old settlers surrounded on all sides by Chukchi and subjected to strong Chukchi influence." Significantly, Tokarev adds that no other use by the Russians of *A. muscaria* is known to him.

Apart from the D'iachkov reference, I found only one other. In a paper by Toren (1970:511) dealing with the use of wild plants in folk medicine, A. F. Gammerman, a specialist in the field, is quoted as having collected data

pangkh to the *A. muscaria*, since linguistically *pangkh* is close to native Siberian words for mushroom. Armless or legless dolls or idols are quite widespread. Among Russians in Vologda guberniia, an armless doll was made at Christmastime to frighten girls and was kept in the front corner of the hut until summer (Ivanitskii 1898:65, fig. 71). An ethnographer working among the Turkic-speaking population of Tomsk guberniia noted the presence of "holy dolls" without arms, legs, or hair which were hung in the corner of the barn; if someone fell ill, the dolls were brought into the house and all Christian objects were turned to the wall (Malov 1909:41). Furthermore, a very important personage in Russian folktales, Baba-Yaga, has only one leg. Laushkin (1970:181–86) points out that the word Yaga probably comes from the Sanskrit word *ahi*, "snake." Originally Baba-Yaga was the Slavic goddess of death, and in many cultural traditions the snake has been the guardian of the underworld—a land peopled by beings both qualitatively and quantitatively different from ourselves (sometimes halflings). The *A. muscaria* too is part of the underworld, although its cap reaches toward heaven. Elizarenkova and Toporov (1970) link the *A. muscaria* with the shaman's world tree, in the sense that both connect the shaman with heaven. It is very tempting to place the *A. muscaria*

within the context of a religious system which contains the following elements: underworld (death), life (fertility), triumph over death (immortality). Whether it is the supreme element in this system (a god) or only one of its components I leave to Wasson to settle. However, A. P. Okladnikov has specifically directed my attention (personal communication, July 3, 1971) to cliff drawings on the Angara and Lena which he has described on several occasions. These armless, one-legged figures, dating from the Glazkov (Bronze Age) period, are similar to figures found in Karelia, the Baltic area, and the Russian North, although the Siberian drawings are noticeably snakelike and the ones from the European areas of the U.S.S.R. are obviously fertility symbols (Okladnikov 1957:45, fig. 12). Some of the figures on the Lena cliffs, however, have both snakelike torsos and crowns like feathers or artichokes (Okladnikov and Zaporozhskaia 1959:95, fig. 34, pl. XXVII, figs. 633, 636, and pl. XXXIX, fig. 792). Dikov (1971:120, Stone X, fig. 81) found a petroglyph in Chukotka which combines *Amanita*-men and the heads of beings very much like those described by Okladnikov. Dikov makes no guess as to the nature of these artichoke-headed creatures, except to say that they are obviously mythological, but he does assume that the *Amanita* men and women pictured on the Pegtymel' River

cliffs are at least in part fertility symbols, masters and mistresses of the reindeer hunted. Archeological data, then, suggest that *A. muscaria* is part of a religious complex. This being so, it is not as important to ask whether a given people used *A. muscaria* or ate mushrooms as it is to ask whether hallucinogens were ritually employed and, if so, to what end. Russian ethnographers have often noted that the Yakuts do not eat mushrooms. The Yakut shaman is not known to have used *A. muscaria*, but he did use tobacco, with almost the same result. (For evidence that tobacco can be hallucinogenic, see Wilbert 1972.) Furthermore, if one is searching the literature for signs of *A. muscaria*, it is of more than passing interest that one description of the Yakut shaman's coat reproduces pictures of it which appear to combine armless (snakelike) good spirits and numerous metallic objects, round in shape, with raised bumps. In the given instance, the ethnographers had no idea what the metallic objects represented (Pekarskii and Vasil'ev 1910:93–116, pl. VI and figs. 2, 11). Later Soviet research, I believe, said that these metallic objects are the sun, stylized by Chinese artistic influence. According to Wasson (1972:201–13) the sun is an important element in the descriptive imagery of Soma.

to the effect that "hunters in the Ural Mountains take pieces of fly mushroom to get drunk and throw themselves into hallucinations."³ I wrote to Gammerman asking for further information about the Urals, and in March 1972 I received this reply:

Information on the use of *Amanita muscaria* in the Urals was given to me by an archeologist who was walking in the woods of the Urals with a local guide of the Zyryan or Komi nationality. In the evenings by the fire, the guide proposed removing fatigue with, instead of vodka, the chewing of small pieces of *Amanita muscaria*. The archeologist said that a real intoxication was achieved, everything seemed colossal—trees were several times thicker, the fire was up to the sky itself, and all the participants behaved better than from vodka. There were no harmful after-effects.

With Gammerman's letter was another, written by a former colleague who had worked for several years in the Far North and Transpolar region of the U.S.S.R. (Inta, Pechora, Vorkuta, Mul'ba, and the settlement of Pro-myshlennyi, Komi A.S.S.R., as well as various settlements of the republic). In the course of his work, which was to collect medicinal plants, he had been given by the Komi a mushroom powder, with the suggestion that he chew it; this he did, ingesting about a tablespoonful over the course of an hour, washed down with birch-fungus tea (*Guonotus obliquus* [Pers.] Br. f. *sterilis*). After about 10 or 15 minutes he experienced a pleasant euphoria that lasted until the end of his day. He experienced a similar euphoria after taking *A. muscaria* on several other occasions in the city of Nar'ian-Mar (Nen National Okrug, Arkhangel'sk oblast) and in settlements along the course of the Pechora River, as well as in the Komi-Permiak okrug of Perm oblast and in the city of Salekhard (on the Ob River). He had known about *A. muscaria* before this time (1946–65) from having seen it in eastern Latvia and western Belorussia and Russia, as well as in Estonia and Lithuania; in these places infusions of *A. muscaria* are used for rheumatic pain.

The medicinal use of *A. muscaria* was interesting, because it was more or less current. In my search for similar information, I found rather frequent references to *chaga*, or birch fungus. There is even a reference on p. 238 of *Soma*, though Wasson's sources call it *zhagra*, and through a mistranslation Wasson overlooks the fact that it was

used as a cure for rheumatic pain.⁴ Since I know from Wasson that birch fungus and *A. muscaria* are frequently found together, I tried (unsuccessfully) to establish a connection between them. I discovered that 19th-century physicians considered the caps of *A. muscaria* the most toxic part and therefore recommended using only the stems in treating a wide variety of diseases: convulsions, paralysis and other nervous disorders, epilepsy, goiter, tuberculosis, congested coughs, scabies and scald-head, rheumatism, intestinal and uro-genital diseases, and various neuroses including, apparently, impotence (Buyal'skii 1859–60:

⁴ Pallas (1967:50–51) reports that the Ostyaks rub the affected parts of the body with birch fungus (*chaga*): "They take a glowing coal and hold it in the vicinity of the pain at various points on the skin, until they find a place where the patient does not feel the burning; then they apply the true burning remedy, which must work and be borne courageously by the patient until the skin is burned through and bursts open." *Chaga* was also used as snuff, rammed into the nose with bast fiber, which plugged the nostrils. The Ostyaks seldom suffered facial frostbite. Krashenninnikov (1949:443, translation mine, with original Russian appended) describes essentially the same method of treatment among the Kamchadaks on Kamchatka, though he calls the medicinal element *zhagra*: "For rheumatic pain they put a birch fungus on the injured places. When this fungus burns to the body it bursts out fiercely and the body decays and there is a great ulcer, on which some people pour fungus ash and some do not treat. And this medicine is known throughout Siberia. (Ot lomu staviat iadna iz berezovogo trudu na bol'nykh mestakh. Kogda onoi trud dogoraet do tela, to otskakiuet s velikim stremleniem, a telo ot togo razgnivaetsia, i byvaet velikaia iazva, k kotoroi inie prisypaiut truduvoi pepel, a inie nichem ne lechat. I sie lekarstvo izvestno po vsei Sibiri)." Wasson, to whom I have shown this paper in manuscript, has consistently maintained that *zhagra* is *Fomes foemularius* (personal communications, May 31, 1971 and May 26, 1972), a judgment supported neither by Dal' (1956, vol. 1:524 [*zhagra*]; vol. 4:438 [*trut*]; vol. 4:580 [*chaga*, *Polyporus ignarius*]) nor indeed by Wasson's source as quoted on p. 238 of *Soma*. Wasson is of course a mycologist of some standing, and he may have access to dictionaries and information unavailable and incomprehensible to me. He defends his translation (personal communication, June 5, 1972) identifying *zhagra* as a pain-killer rather than as a cure for rheumatic pain on the grounds that the word *lomota* is used for every sort of joint pain. The commentary to Krashenninnikov (1949:236 n. 1) says that birch fungus is used "v kachestve sredstva ot lomoty." Chester Chard (personal communication, July 13, 1972) points out (as does Pallas, but in the edition I used the word was misspelled) that "burning the birch fungus . . . is a form of *moxa* cautery which was practiced (with various substances) from Lapland to China and Japan. Still is." My dictionary defines *moxa* as a counterirritant, but this is not *chaga's* sole function, as Min'ko (1969) indicates. As Chard also points out, *chaga*

487–92).⁵ It was recommended that the *A. muscaria* be dried in the oven and preserved in a stoppered jar in a dry place. *A. muscaria* was a folk remedy in Novgorod guberniia for rheumatism, swellings, and periostitis (the cause of which was thought to be a cold) (Gerasimov 1898:126, translation mine):

They collect mature specimens of the mushroom, breaking them into pieces, putting them in a bottle filled to the top; they stop it up and leave it in a warm place for three days. Under the influence of the heat, one receives a semi-liquid mass, and they rub with the crust. In one case, known to me, with frequent and severe rubbing of the leg (a very severe *Ischias*) [*sic*], a poisoning occurred, expressed in vomiting and pain in the stomach, but the pain in the leg decreased.

A somewhat similar remedy was recorded as in use among the Lithuanians, except that *A. muscaria* was only one of several remedies used (Petkevich 1911:209). In her letter to me, Gammerman cites a handbook of medicinal plants published in Minsk in 1965. It lists as components of *A. muscaria* muscarin, muscaridin, and a red antibiotic substance called muscarufin. It is this substance which is thought to act on tumors. The same source notes the use in Belorussian folk medicine of the *A. muscaria* liquid for rheumatism, but, Gammerman writes,

has a prominent place in the folklore of the patients in Alexander Solzhenitsyn's *Cancer Ward*. On p. 278 of *Soma*, quoting Bogoraz (1899), there is a Chukchi riddle: "I have a headache. I am bleeding from my nose. Stop my nose bleeding! . . . What is it?" Answer: "Fly agaric." Botoraz seems to think that this is because eating *A. muscaria* causes a violent headache. Other sources do not appear to support this conclusion. Vinogradov (1915:410) says that *chaga* was used by the East Siberian Russians as a cure for headache. He also notes that the birch fungus *Polyporus* sp. was used in a manner which his informant did not wish to discuss. Another remedy (Vinogradov 1915:403, item 36) is a fungus (*gryb*) which seems to grow in basements. Its use is the same as that described for *A. muscaria* for rheumatic pain and swelling of various sorts, except that it is mixed with butter. Wasson (personal communication, June 5, 1972) insists that *chaga* and *A. muscaria* are not used for the same purposes, although he also admits that "both are bathed in what anthropologists call *mana*. . . ." I do not think that the connection between the two substances can be disregarded, nor should the possibility be excluded that Bogoraz did not know everything there was to know about the use of *A. muscaria* among the Chukchi. The point at issue here and elsewhere is whether a given people will use a certain substance (either ritually or medically) if another (more common, more easily available, more reliable) is known to them and used for the same purpose.

⁵ Buyal'skii seems more concerned with the fact that sheep pastured in meadows with *A. muscaria* do not suffer from worms.

³ Toren also says that the *A. muscaria* is used against stomach ache and other illnesses: "The people drink its sap, which, used in large quantities, acts as a soporific."

there is no internal use. However, it is worth noting the following passage from a book on Belorussian folk medicine (Min'ko 1969:82–83, translation mine):⁶

For the treatment of gastritis, ulcers, and stomach cancer, they use the *chaga*, a black birch fungus, *Fungus betulinus*. Infusions and decoctions are used in folk medicine today in Minsk, Vitebsk, and Brest oblasts. The following mixture is used for cancer of the stomach: the birch fungus, the flower of bitter aloe and wine. All this is boiled and used in one tablespoonful several times a day.

At present the *chaga* is used for the treatment of gastritis, ulcers, and malignant tumors. According to experimental data, it has no destructive action on the cancer cells but improves the patient's sense of well-being, and in the beginning stages can inhibit the growth of the tumor. It is characteristic that for cancer the Belorussians use every kind of fungus, starting with the *chaga* and the white *borovik* [*Boletus edulis*] to the growths on apples.

Wasson, to whom I have reported this, dismisses it as nonsense, as he does references to the chemical composition of *A. muscaria* in textbooks, saying that the handbooks are hopelessly out of date. In fact, Gammerman could report to me no current research on *A. muscaria*. Interest of a sort may still be high, if we can judge by the fact that a Soviet public-health journal with an edition (for that number at least) of 262,769 copies carried an article on symptoms and treatments for mushroom poisoning: four paragraphs are devoted to *A. muscaria* (Lokai and Bogachik 1971:21). From the layman's point of view, it is interesting that the patient's reaction is said to vary, depending on whether muscarin (described as a depressant) or muscaridin (described as a stimulant) predominates. Whatever the merits of

the article, it does give a clue to the reason little research is being published on *A. muscaria* in the Soviet Union today: as a drug, it is quite unreliable.

Nevertheless, I think further research into the use of *A. muscaria* in folk medicine would be of some value. Wasson believes that not enough muscarin can be isolated in the *A. muscaria* mushroom to account for its hallucinogenic properties (personal communication, September 3, 1971),⁷ and both the literature and individual testimony emphasize the fact that reactions vary considerably. If D'iachkov believed that three *A. muscaria* could intoxicate, more experienced users have been known to ingest as many as 21, though this is the outer limit (Patkanov 1891:89).⁸ Under the circumstances, there may be some justification for assuming that it is not the *A. muscaria* itself but the context of its use that intoxicates.⁹ *A. muscaria* was and perhaps continues to be widely used in folk medicine. Folk healers frequently accompanied prescribed cures with a spell or incantation. These incantations are magical in intent, but the poetic imagery is frequently religious, with Christian and pagan symbols mixed (see Kapustin 1971:55 n. 5; also Min'ko 1971).¹⁰ Although *A. muscaria* is an effective remedy in some cases, its effect is far from consistent. Can it be that it is employed out of some feeling that it is a sacred substance, as, within a Christian context, water and oil are sacred?¹¹ Clearly,

⁷ An encyclopedic source quoted to me by Gammerman indicates that although there are only about 0.018 g of muscarin in 100 g of fresh *A. muscaria*, only 0.005 g are necessary to produce signs of poisoning; these symptoms can be accompanied by hallucinations (*Entsiklopedicheskii slovar' lekarstvennykh, efirno-maslichnykh i iadovitykh rastenii*, Moscow, 1951, pp. 244, 315).

⁸ It is worth noting that 21 is a multiple of 7, a magical number, and that 3 is also a magical number. In this instance, the *A. muscaria* was used to improve the singer's memory and his endurance. Under the *A. muscaria*'s influence, he could sing long-forgotten *byliny* for an entire night.

⁹ Furst (1972:xiv–xv) seems to apply this hypothesis to a wide variety of hallucinogens.

¹⁰ Min'ko somewhat overstates his case against homeopaths (and contradicts his earlier book) by claiming that the word (the incantation) is considered more powerful than the remedy itself. He does not specifically mention *A. muscaria*.

¹¹ All the medicinal uses of *A. muscaria* I have been able to find appear to rely on its ability to "draw out" pain. Shamans frequently took *A. muscaria* before attempting to heal a patient because, apparently, their principal function was to extract the pain-causing element. Zelenin (1936:344) says that idols were fashioned to assume the patient's particular illness. Remnants of this belief can be found, he says, among

more research along these lines would tell us something about the nature of religion and the religious experience. I can only agree with Wasson that the reason so little is known about the *A. muscaria* is that cultural anthropologists have neglected to ask the right questions.

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Traditional Use in North America of *Amanita muscaria* for Divinatory Purposes

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Until now no inquiries into the role of fungal hallucinogens in prehistory have ever dealt with the natives of North America. This has not meant that hard evidence was lacking: that evidence, though scant, has been accessible to any diligent seeker. To most it will come as a surprise to learn that natives of this land shared with the Eurasians the fly agaric (*Amanita muscaria*) practices documented in *Mushrooms Russia and History* (Wasson & Wasson 1957) and more fully in *Soma: Divine Mushroom of Immortality* (Wasson 1968). Only the role of the reindeer in the Siberian mushroom complex is perforce absent from the American usage.

Evidence is available that indicates the Ojibway and some others of Algonkian linguistic stock living in the woodlands of this country and Canada consulted the fly agaric for divine guidance in their shamanic observances. This evidence will help in clarifying the ambiguities and murky passages of Siberian informants. May not our new testimony from the New World and the Siberian documentation, taken together, suggest a common ancestry in the remote past? Just as in Siberia, here also there was use of the mushroom for its inebriating potency. We must not forget that north of Mexico most of our native population before the white man came possessed no alcoholic beverages and the only road to inebriation lay in ingesting plant hallucinogens, amongst which the fly agaric was conspicuous.

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Almost 20 years ago, shortly after the publication of *Mushrooms Russia and History*, Claude Lévi-Strauss, the famous anthropologist, wrote us a letter from Paris calling attention to a remarkable citation concerning mushrooms in the *Jesuit Relations*. A Superior of the Jesuit Order, Père Charles l'Allemand in a lengthy epistle dated 1 August 1626 from Quebec to his brother in France, also a Jesuit Father, undertook to describe the customs of the natives thereabouts, an Algonkian group. Discoursing on their religious beliefs he wrote (and I translate):

... they assure you that after death they go to heaven where they eat mushrooms and hold intercourse with each other.

He fails to tell us why they singled out mushrooms to eat, nor does he tell us what kind they ate. We can now say with certainty that those mushrooms were *Amanita muscaria*. Let it also be noted that Père l'Allemand's letter to his brother was dated 1626, a full century earlier than the earliest reference to the Siberian mushroom practice, viz. von Strahlenberg's few words published in 1730.

For years I was busy with mushroom problems in far parts of the world but this citation that my friend Lévi-Strauss had sent never ceased to rankle in me. I simply had to find time and opportunity to follow it up. But I did not happen on anyone in touch with Algonkian speakers until the spring of 1975 when I found myself seated in the Harvard Faculty Club next to Yves Goddard. He was lecturing at that time in the Linguistic Department on Amerindian languages and

specializing in the Algonkian family. When he told me his specialty, I quickly asked what this group's attitude was toward mushrooms. Surprised, he said he had never thought to ask but he manifested the liveliest interest. He suggested I write to John Nichols lecturing at the University of Wisconsin. This I did on 26 April. Nichols replied most courteously by return mail that he knew nothing about my subject but he gave me the address of a "native speaker" who had prepared a paper on mushrooms. This "native speaker" was Keewaydinoquay, the sole surviving person living on a small island in the waters of the Great Lakes area where once there were scores of her people. In her 60s, five-eighths Ojibway, she is herbalist and shaman of the Ojibway tribe. She is a fierce defender of the rich cultural legacy that comes down to her from Ojibway ancestors.

This contact with Keewaydinoquay has proved richly rewarding. We have exchanged lengthy letters and I found that through her the portals were opened for me into Ojibway manners and traditions. She has visited me in Connecticut on three occasions. In August 1976 I spent a week on her island and there she presented to me a superb and precious birchbark scroll. In picture writing she told the legendary tale of the origin of the fly agaric discovery among the Ojibway and the deep-seated ambivalence of this race toward it. In August 1977 my colleague Reid Kaplan passed a week on her island and Kee initiated him into the rites of the formal *miskwedo* ceremonial.

The deep-seated and sometimes violent ambivalence toward the fly agaric among the Ojibway is documented not only in the scroll that Keewaydinoquay has given us — one of the dramatic events in her own life also embodies it. She was apprenticed as a child to an herbalist, Nodjimahkwe and served her apprenticeship with this remarkable woman for a number of years. Nodjimahkwe was strongly, even fanatically, opposed to the use of *Amanita muscaria* and, although Keewaydinoquay respected and loved her teacher, staying in close contact with her for a number of years after her apprenticeship, she learned the art and technique of taking the sacred mushroom from another herbalist, also respected in the highest degree. We gather from the clear evidence of the scroll that this ambivalent attitude of the Ojibway to the sacred mushroom is an ingrained element in Ojibway culture stemming far back in time. From our Siberian witnesses we get no hint of a similar ambivalence there, but we must allow for the possibility, even likelihood, that our informants simply failed to elicit evidence concerning it.

The Ojibway lived and live in what is now Michigan, Minnesota, Wisconsin and the adjacent regions of

Canada. They were renowned for the many uses to which they put birch bark and perhaps the most notable and distinctive of these uses was the *ojibeweg*, the hand-drawn picture writings on scrolls of birch bark. They had developed this art of picture writing much further than any others of the races that dwelt in North America. According to Kee, it is from this word *ojibeweg* that we have taken the name by which the Ojibway are commonly known. Though it seems to me that this name is a compliment to her people, Kee objects to it and says they should be called by the word that they use in speaking about themselves, "Ahnishinaubeg," and in deference to her wishes I shall hereafter use this name. The Ahnishinaubeg have many names for the fly agaric, among which the most common today is the *miskwedo*, the "redtop," although this English rendering lacks one important element inherent in the term *miskwedo* — that it is a mushroom dependent on trees.

Kee drew our picture scroll from memory of a scroll she saw in possession of a story teller who lived long ago. This story teller bore the name Ispákwe, the "Spanish woman." (Ispákwe, incidentally, was renowned for her uncanny skill in separating the successive layers of the birchbark with which the scrolls were made.) We must remember that in all unlettered cultures those charged with being the culture-bearers trained their memories to an astonishing degree. These picture scrolls are mnemonic aids to the human memory, in this case to the story teller's memory as he or perhaps she retells the old, old story of the *miskwedo*, embellishing the tale with grace notes, intonational flourishes, and the beating of the small drum (a *pim* drum) that accompanies the telling. The time for the story telling was the long winter evenings and nights, when old and young of both sexes would crowd together in the wigwam around the fire and the fame of the good story teller would spread far and wide. Though perhaps 50 years had passed, Kee had little difficulty in giving us the *ojibeweg* of the *miskwedo* tale: a fitting performance for one belonging to the Crane Clan as she does. The scroll of which I am now the proud owner traces its lineage to Ispákwe but its more remote lineage, untraceable, goes back, back, how far back no one can say. It is our incredible good fortune to have arrived on the spot in time to profit from the presence of Keewaydinoquay who, apart from much else, has given us this *ojibeweg* of her people, the mushroom tale of the Ahnishinaubeg. Let us remember, however, that the *ojibeweg* are far more than mnemonic scrolls carefully kept in hollow tree trunks to preserve tribal traditions. Those who are masters of the *ojibeweg* can write anything with them: letters to absent ones;

notes for friends to pick up; etc. The scrolls carefully kept in museums and universities give undue emphasis to documents written for lasting use.

We have confirmation from outside sources of the elements of this tale up to the final section that Reid Kaplan and I have come to call the Coda. The publication of the scroll may bring out from Norval Morrisseau or others an affirmation that this part of the story conforms to their understanding.

How has the *miskwedo* escaped attention until now? The Catholic missionaries for centuries were excommunicating natives who resorted to the mushroom; Protestant missionaries similarly condemned its use. In a series of episodes that Keewaydinoquay has supplied us with, we find that the Whites living in daily contact with the Ahnishinaubeg knew of the *miskwedo* and sometimes invoked its aid or asked a native to do so on their behalf. In the Tenth Annual Report of the Bureau of Ethnology (1888-1889), we discover this startling sentence concerning the Ahnishinaubeg:

The ceremonial chants could be advantageously pronounced only under inspiration, *which was of old obtained by a tedious form of innoxication*, now expedited by alcohol. (Italics added)

What was this unexplained "tedious form of innoxication?" The ceremonial use of *miskwedo* of course. It seems that everyone — everyone except the anthropologists who I am told prefer to study native peoples in distant lands, and the mycologists, of course, who in Michigan since the days of C.H. Kauffman have held themselves in rather high regard — knew of this deeply ingrained religious practice of the Ahnishinaubeg. Perhaps it is just as well, as the ceremonial use of *miskwedo* has been preserved for us as in a time capsule, unsullied by mishandling.

We have no way of telling how far back the ancestry of our scroll goes nor how far afield. Thanks to Kee, we possess ample documentation on the Ahnishinaubeg, and in the light of Kee's testimony, the meager evidence for the use of *miskwedo* among the Montagnais and Abenaki — Algonkian speakers, both of them — takes on persuasive significance. But is it conceivable that they alone, of the native peoples of North America, had recourse to the Divine Mushroom of Immortality? How about the Lenape, a woodland people of the East Coast,

who also lived where the birch and the conifer flourish, and all the other Algonkian-speaking tribes? And how about the Iroquois and their kindred peoples? And the peoples on the Northwest Coast where the *miskwedo* abounds? And the Athabascans on the Upper Yukon?

Perhaps it is advisable that I remind the reader of our utter dependence in our own culture on the alphabet. Our alphabet, supplemented centuries ago by the printing process, in recent decades by our photocopy devices and even more recently by our retrieval systems, shapes and molds our lives, for better or (possibly) for worse. In Kee, with her immense heritage of oral culture, we have an exemplar of the old culture that existed — and indeed still exists — for far, far more millennia than the brief five or six that comprise the life of the written word. Early man, by which term I mean our ancestors before they possessed the faculty of writing down what they said, relied on human memory for their knowledge of the past, and in those who were the culture bearers that memory, perhaps a little inaccurate, was prodigious. Kee is a singularly able exponent of both cultures — heir to the legacy of oral culture from her native people and practitioner of our contemporary American culture.

Reid Kaplan and I now have the unusual privilege, a privilege that we hardly dared hope for, of presenting to you today in person an Ahnishinaubeg Shaman, Keewaydinoquay, member of the Crane Clan, the Clan that is the repository of the knowledge and traditions of her people. She speaks and writes English better than many of us. At first and for a long time she refused even to consider coming here: she was unwilling to discuss the religious beliefs of her people in front of a large and decidedly mixed gathering. I have assured her that this gathering, though indeed large and mixed, is thirsting for what she alone can convey to you and that you would all understand her position as one who knows well the *miskwedo*, who took the *miskwedo* for the first time at the age of about 14, who has taken it three or four or five times in almost every year since then, over close to a half century, and who knows that when taken in grave situations and in purity of heart, it gives inspired guidance. She has never taken the *miskwedo* lightly or frivolously or "for recreation."

And now I present to you, Keewaydinoquay, who will tell her story, the story of the scroll.

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SOMA AND AMANITA MUSCARIA

By JOHN BROUGH

The importance of the Soma-plant in Vedic religion has never been underestimated. Among the rituals of the Yajurvedic texts, the *soma*-sacrifices are among the most elaborate and important, and are described in minute detail in the *Brāhmaṇas* and *Śrauta-sūtras*. These later texts nevertheless continue a direct tradition from the *R̥gveda*, which can be seen to reflect an earlier stage in the development of the ritual, doubtless of a less rigidly formalized and probably less elaborate nature. Even so, the *R̥gveda* is, so to speak, permeated by Soma. Understandably, therefore, from the early days of Vedic studies in the West, many scholars have speculated on the botanical identity of the plant in question. Numerous candidates have been nominated, the most frequently favoured being species of the genera *Ephedra*, *Sarcostemma*, *Periploca*, and latterly *Cannabis*, and even *Rheum*. Not a single one of these conjectures has gained general assent, and the opinion is widely held that the problem is insoluble.

Earlier, *soma* was usually thought to be a fermented drink ; but the facts of the ritual exclude this, and most scholars would now reject the alcoholic theory. It is then easier to understand the sharp contrast between the divine *soma* and the presumably fermented *surā*. The latter drink is frequently mentioned with disapproval in the later Vedic texts : for detailed references, see P. V. Kane, *History of dharmaśāstra*, II, 792 ff. It is fair to add that *surā* seldom occurs in the RV, and in this earlier period only its excessive consumption seems to be considered bad. The use of *surā* in the Sautrāmaṇi, and in conjunction with *soma* in the Vājapeya, is thus not necessarily paradoxical.

The situation is comparable in the Avesta : *Yast* 17.5 (and similarly *Yasna* 10.8) *nəmō haomāi yaθ vīspe anye maδāñhō aēšma hačinte xrvīdvrō, āaθ hō yō haomahe maδō aša hačaitē xʷaēpaiθē* ‘Homage to Haoma, in that all other intoxications are accompanied by Frenzied Wrath with bloody club, while that intoxication which is Haoma’s is accompanied by his own *Aša*’. By implication, this would include *hurā* among the other ‘bad’ drinks ; but specific references to *hurā* in the Avesta are regularly neutral, and it is mentioned merely as a drink, without condemnation. Similarly in Middle Iranian, Pahlavi *hur*, a drink for kings and nobles : see W. B. Henning, *BSOAS*, xvii, 3, 1955, 603.

In RV 7.86.6 *surā* is one of several causes of sin against Varuṇa ; it is linked with *soma* in 8.2.12, perhaps implying that the two drinks had some effects in common : *hr̥tsū pītāso yudhyante durmādāso ná sūrāyām* ‘When consumed, they (the *somas*) fight in the heart (of Indra?) like men badly inebriated on *surā*’ ; while the gift from the *Aśvins* to *Kakṣivant* of a hundred jars of *surā* is obviously a splendid blessing in 1.116.7 *kakṣivāte . . . śatām kumbhām asiñcatam sūrāyāh* ; and this is obviously the same blessing or a closely similar one accorded to the tribe (*jānāya*) of *Kakṣivant* by the *Aśvins* in the next hymn, 1.117.6 *śatām kumbhām asiñcatam mādḥūnām*—‘of intoxicating drinks’ rather

than 'of honey'. It will be convenient to postpone discussion of this last point until later in this article.

It is thus natural, in the quest for the nature of *soma*, to turn to the consideration of a vegetable alkaloid or similar substance.

Discussion of the identity of the Soma-plant has latterly been in the doldrums. It is therefore greatly to the credit of Mr. R. Gordon Wasson that he has re-awakened interest in the problem by the publication of a detailed and scholarly investigation, propounding the revolutionary theory that the Soma of the *Rgveda* was a mushroom.¹

'My candidate for the identity of Soma is *Amanita muscaria* (Fr. ex L.) Quél., in English the fly-agaric, the *Fliegenpilz* of the Germans, the *mukhomor* of the Russians, the *fausse orange* or *tue-mouche* or *crapaudin* of the French, the brilliant red mushroom with white spots familiar in forests and folklore throughout northern Eurasia.

'This is the first time that a mushroom has been proposed in the Soma quest.

'The fly-agaric is an inebriant but not alcoholic. As far back as our records go, it has been the Sacred Element in the shamanic rites of many tribes of northern Siberia, tribes that are concentrated in the valleys of the Ob and the Yenisei, and then, after an interruption, other tribes in the extreme northeast of Siberia' (p. 10).²

In summary, Wasson considers that the Soma-cult is explicable as an Indo-Iranian representative of a fly-agaric cult which in early times was wide-spread throughout a great part of Eurasia: 'How astonishing that we can still draw parallels with the fly-agaric cult in Siberia, where as we shall see in *Part Three* it lingers on, in the last stages of degeneration among the peripheral tribes of the extreme north' (p. 68). He therefore devotes a major part of his book to the discussion of the use of the fly-agaric and other mushrooms in lands beyond India and Iran, and especially in Siberia. These sections of the book are full of interest as an aspect of cultural history; but as a matter of simple logic, such parallels have no probative value. They cannot even be adduced as confirmatory arguments for the theory that the Soma-plant was *A. muscaria*. Until this theory is proved for the *Rgveda*, and proved beyond any possible

¹ R. Gordon Wasson, *Soma, divine mushroom of immortality*. (Ethno-mycological Studies, No. 1.) xiii, 381 pp., 24 plates, 3 maps. The Hague: Mouton; New York: Harcourt, Brace and World, Inc., [c1968] (publ. 1969), \$200 (£86.50). The book is sumptuously produced, printed on hand-made paper specially water-marked, with 24 colour plates. Edition limited to 680 numbered copies. Mr. Wasson's primary study is mycology, and he was for 10 years a Research Fellow of the Botanical Museum of Harvard University, now Honorary Research Fellow; also Honorary Research Associate and former member of the Board of Managers of the New York Botanical Garden. In my discussion of the theory here, I am much indebted to Professor Mary Boyce and Dr. I. Gershevitch for information and advice on some of the relevant Iranian materials. These colleagues are of course not necessarily committed to any of the views which I have expressed; and any inadvertent errors are my own.

² Throughout the present article, such references, unless otherwise indicated, are to pages or plates of Wasson's book.

doubt, the non-Indo-Iranian materials remain, in the strictest sense, irrelevant. Even if the proposed identification for Soma seems probable, but is not proved on the basis of internal evidence, extraneous facts are not additional evidence. On the other hand, if the Vedic case were proved, we should indeed begin to consider the possibility of a very wide-spread and significant cultural religious continuum.

The present article is therefore mainly concerned with the first section of Wasson's book, namely, that which gives his detailed arguments from the text of the *R̥gveda* itself. Because the book is costly, and the edition limited, it may be useful to readers to prefix a summary of the contents of the remainder of the book.

The main argument is presented in pp. 3-70, where are included many splendid colour photographs of *A. muscaria* to illustrate aspects of the plant which, as Wasson believes, inspired the epithets and tropes applied to Soma by the poets of the *R̥gveda*. He excludes later texts, on the grounds that the original plant had already been replaced by substitutes, and was possibly in process of being lost even by the time of the later hymns of the RV : 10.85.3 *sómam yám brahmāṇo vidūr ná tasyāśnāti kás caná* 'The Soma that the Brahmans know—that no one drinks' (quoted and translated, p. 14). The remainder of part I consists of 'Mani, mushroom, and urine' (ch. xii, pp. 71-6); and 'The marvelous herb' (ch. xiii, pp. 77-92)—a brief passage from the *Shāhnāma*, another from the *Padma Purāṇa*, and a longish discussion on the famous *ling chih* 靈芝 of the Chinese, recognized to be a different mushroom, at least in part mythical, but believed by Wasson possibly to have been inspired in part by tales of a mushroom cult brought to China by way of Central Asia.

Part II, 'The post-Vedic history of the Soma plant' (pp. 95-147), is contributed by Dr. Wendy Doniger O'Flaherty. This is a useful essay which starts by bringing together statements about Soma in the *Brāhmanas*, *Śrauta-sūtras*, and later Sanskrit works, including prescriptions of possible substitutes for the Soma-plant when the latter is not available. The substitutes, as would be expected, are only Sanskrit names to us, and few if any can be identified botanically. There follows an interesting section giving a history of the controversies on Soma in the nineteenth and twentieth centuries; and it is instructive to observe not only the multifarious plants proposed, but also the quite superficial approach of the majority of the writers on the subject.

On p. 97, 'the *nyagrodha* (sacred fig or banyan tree)', and p. 122, 'the *nyagrodha* (*Ficus religiosa*)', is a mistake. The *nyagrodha*, *vaṭa*, the banyan, is *Ficus benghalensis* L. (given in the Sanskrit dictionaries as *F. indica* L., a discarded name for the same plant: it is now an error to use '*F. indica*'). *F. religiosa* L. is the *aśvattha*, *pippala*.³ This may be an isolated lapsus; but it suggests that the numerous botanical names and 'synonyms' given in this chapter

³ The Sanskrit dictionaries are notoriously unreliable on botanical nomenclature. Among the most frequently mentioned figs in Sanskrit, only *Ficus religiosa* = *aśvattha* still stands as a valid name, while others continue to be miscalled by names now discarded by botanists under the

may sometimes require further taxonomic research before they may be safely quoted.

In part III, 'Northern Eurasia and the fly-agaric' (pp. 151-204, followed by an epilogue on 'The tree of life and the marvelous herb', to p. 222), Wasson resumes the tale. In the concluding section of the book, entitled 'Exhibits' (pp. 233-356), extracts are quoted in English (translated from other languages as needed) of passages relevant to the fly-agaric from the writings of explorers, travellers, and anthropologists. The earliest of these passages is from 1658, but the majority are much more recent. Annotations are added where necessary, and Wasson is careful to indicate points where the witnesses are unreliable. Most of the 'Exhibits' concern Siberia; but in dealing with the last few, on Scandinavia, Wasson rejects the theory that the old Norsemen went 'berserk' through consuming the fly-agaric. This would have been a good parallel to the stimulation for battle which Indra obtained from *soma*. But the Norse tale of the fly-agaric in this connexion first appears in the late eighteenth century, and is thus without value.

As already indicated, Wasson's arguments directly drawn from the RV are contained in the first part of the book, the detailed evidence being concentrated in less than one-fifth of the volume. To these arguments we now turn our attention.

It must be emphasized that Wasson did not deliberately set himself to identify the Vedic Soma. In an earlier book⁴ he and his wife 'explored the folkloric and linguistic background of the fly-agaric throughout Europe, and showed the deep hold that it exerted at one time on the imagination of the north European peoples' (p. 35). It was only much later that he became acquainted with the RV, through the translations of Geldner, Renou, and Bhawe,⁵ and gradually came to the conviction that 'the hymns of the RgVeda fit the fly-agaric like a glove. True, one must possess some awareness of the psychotropic

priority rule. The opportunity is taken here to correct some of these: for further details, see E. J. H. Corner, 'Check-list of *Ficus* in Asia and Australasia', *The Gardens' Bulletin* (Singapore), xxi, 1965-6 (publ. 1967).

udumbara : *F. racemosa* L. [wrongly *F. glomerata* Roxb.]

udumbari, *añjīra* (Pers. انجیر) : *F. hispida* Linn. f. [wrongly *F. oppositifolia* Roxb.]

parkaṭi : either *F. virens* Ait. [wrongly *F. infectoria* Miq.]

or *F. tsjahela* Burm. f. [wrongly *F. infectoria* Willd.]

or *F. caulocarpa* Miq. [wrongly *F. infectoria* Willd. var. *caulocarpa* (Miq.) King].

This last is a good example of the confusion which can arise when, as is usual in Sanskrit dictionaries, the naming authority is omitted: s.v. *parkaṭi* we find only *F. infectoria*, and are thus left with three possible interpretations.

⁴ R. G. Wasson and V. P. Wasson, *Mushrooms, Russia and history*, New York, 1957.

⁵ Karl Friedrich Geldner, *Der Rig-veda aus dem Sanskrit ins Deutsche übersetzt* (Harvard Oriental Series, xxxiii-xxxv), Cambridge, Mass., 1951; Louis Renou, *Études védiques et pāṇinées* [EVP], especially Tomes viii and ix, Paris, 1961 (translation with notes of the ninth *maṇḍala*, to Soma Pavamāna); S. S. Bhawe, *The Soma-hymns of the Rgveda*, Baroda, 1957, 1960, 1962 (containing 9.1-70).

plants of the world and their rôle in primitive religion. Given that familiarity, a reading of Geldner, Renou, and Bhawe leads straight to the fly-agaric ' (p. 67).

The argument depends chiefly on the assumption that many of the descriptive phrases applied to Soma in the RV are direct descriptions of the growing mushroom, or poetical figures suggested by it at various stages in its development. Much persuasiveness is added by the brilliant colour plates ; and since these cannot be reproduced here, Wasson's own account of *Amanita muscaria* (p. 35) is quoted as an aid to clarification.

' In the fall of the year, hard by a birch or pine, one is apt to find the fly-agaric. The season in the temperate zone lasts two or at most three weeks, with the climax coming in the middle week. The fly-agaric emerges like a little white ball, like cotton wool. It swells rapidly and bursts its white garment, the fragments of the envelope remaining as patches on the brilliant red skin underneath. At first the patches almost cover the skin, but as the cap expands they are reduced in relative size and finally are nothing more than islands on the surface. In fact, under certain conditions, especially as a result of rain, they are washed off altogether and the fly-agaric then shines without blemish as a resplendent scarlet mushroom. When the plant is gathered it soon loses its lustre and takes on a rather dull chestnut hue.'

It is to this plant in its various stages that, in Wasson's opinion, the hymns of the RV refer, in the epithets and tropes which they apply to Soma. ' It is certain that the poets of the R̥gVeda knew the original Soma at first hand, and they never strayed from it for long ' (p. 12). But is it certain? It is difficult to imagine that the ritual utterances of the ceremonies of Soma Pavamāna (the examples are naturally taken mainly from the ninth *maṇḍala*) should be dominated by rapturous descriptions of *A. muscaria*, a plant which can be seen in its beauty during only a few days of the year. The dull, dried specimens which must have been used at almost every sacrifice—and invariably after the Vedic people had reached the plains of northern India—could hardly have inspired poetic rapture. At the pressing and clarifying, the priests are intent on the ritual situation ; and it is far more likely that the rapture is to be attributed to the remembered and anticipated psychotropic effects of the *soma*-juice. Granted that, unlike *soma*, opium is not sacrosanct, it is scarcely likely that a user of opium would rhapsodize over the beauty of the flower of *Papaver somniferum*.

This comparison may perhaps seem a little unfair, when the priests were preparing the drug from actual plant material during the rite. Still, the accompanying hymns are essentially ritual utterances. We cannot deny the possibility that the hymns could reflect at least some features of the wild plant ; but in the context of the ritual pressing and filtering of the *soma*-juice, it seems hazardous to conjecture that such reminiscences could have been so omnipresent as Wasson would have us believe. It is a delicate task to interpret passages from hymns which can involve the plant, or the pressed juice, or the deification of the latter, the god Soma. The poets draw no sharp distinctions

between these three. Even if the deified Soma may still show some characteristics based on the natural plant, mythological accretions are expected, and frequent. Often, details of the ritual acts, the divine Soma, and the physically present *soma*-juice so interact in the minds of the priests that an attempt to analyse these aspects could only distort the hieratic purport of the poets.

Wasson is not unaware of this, although in my opinion his arguments give undue prominence to his fugitive natural plant. 'Soma was at the same time a god, a plant, and the juice of that plant' (p. 3). This statement requires modification. No one has doubted that *soma*, in fact as well as by etymology, is the pressed juice, and that it is this juice which is the original 'place of manifestation' (*dhāman*, on which see below) of the deity Soma. So far as it is possible to be definite in such matters, the word *soma* seems never to be used directly as the name of the plant, unless perhaps occasionally as a metonymy (and even then the reference may be to the indwelling deity). One verse shows that this was clear to the Vedic poets :

RV 9.92.2

āchā nṛcākṣā asarat pavītre

nāma dādhānaḥ kavīr asya yónau :

'(Soma) . . . flowed hither, taking to himself his name on the filter, on his *yoni*'.⁶ When Soma is said to be located on the mountains, it seems almost certain that the sense intended is that the deity Soma is present in the sap of the plant. It is therefore probably going too far to say that 'Soma is the only plant that man has ever deified'. Rather, the situation is comparable to that quoted (p. 3) for the Mexican Indians, who 'seem to regard the hallucinogenic plants, whether mushrooms, *peyotl*, or morning glories, as mediators with god, not as a god'.

We thus have the peculiarity that a plant of unique importance in Indo-Iranian religion seems never to be named. Since the whole of the Veda was a secret text guarded by the priests, there is no reason to suppose that a taboo was responsible for this.

In the RV, references to the *aṃśu-* of Soma are frequent, the word being traditionally rendered as 'stalk' or 'stem'. Renou, for example, has 'la tige' or 'la tige (de *soma*)'. There seems to be no reason or indication from the text itself for the choice of the stalk. Indeed, if Wasson were right, it would be all the more extraordinary that the cap of his mushroom, the cap which provides so many details for his argument, should be thus discarded when the plant comes to be pressed in the ritual. (Wasson admittedly identifies *mūrdhan*, *śiras* 'head', when used in connexion with Soma, with the cap of the mushroom (pp. 45-6); but this interpretation is unconvincing : see below.) In the numerous Siberian examples quoted in the 'Exhibits', the mushroom is apparently always consumed whole. For the RV, we might guess that the potent drug was concentrated in the stalk of the Vedic plant, or even that other parts were noxious. There are no grounds for believing that the Indo-Iranians were sufficiently skilled herbalists to have made such discoveries.

⁶ For *nṛcākṣāh*, Renou, ' (le *soma*) au regard de maître ' ; Geldner, ' Der männlich Blickende '.

On reading through the Soma-hymns, the recurrence of *aṃśu* is striking. One gradually comes to suspect that the word does not mean 'stalk', and the conviction begins to grow that we have here the actual name of the plant itself.

In seeking to verify this suspicion, a belated reference to Grassmann's dictionary showed that this idea was not new.⁷ Under the word, Grassmann gives : 'Name der Pflanze, aus welcher der Soma gepresst wurde. . . . Also : 1) *Somapflanze*, 2) der aus ihr gepresste *Somasaft*'. An examination of all the passages in the RV where the word occurs confirmed that in every relevant⁸ instance, Grassmann's interpretation did no violence to the sense, but on the contrary usually fitted more naturally in the verses in question. In 1.46.10 *ābhūd u bhā u aṃśāve*, Geldner actually translates 'Licht ist der Somapflanze geworden'; Renou (*EVP*, XVI, 5) '... est apparue la lumière (adaptée) au soma'. Elsewhere, Geldner always gives 'Stengel' for *aṃśu*, with one exception, 9.15.5 *eśā . . . śubhrēbhīr aṃśúbhiḥ* 'Dieser [Soma] . . . mit den . . . strahlenden Zweigen (Strahlen)', with the footnote '*aṃśúbhiḥ* dann doppel-sinnig'. Renou in a note on the same verse agrees that here '*aṃśu* a pour acception seconde "rayon"'. In spite of the occasional use of *śubhra* as an epithet of Soma (not explicitly of *aṃśu*), it seems risky, on the basis of this solitary verse, to read into the RV the common later Sanskrit sense of 'ray'.

It is slightly quaint to find both Roth and Renou⁹ mentioning *aṃśu* as the name of the plant, and immediately adding—almost as an afterthought—that, strictly speaking, the word meant the stem : 'Die dem Veda so geläufige Bezeichnung für die Somapflanze oder vielmehr ihre Glieder'; '*Aṃśu*-désigne, aussi bien dans le RV. que dans l'Avesta, le soma en tant que plante (et proprement la tige ou les fibres du soma)'.¹⁰

Roth (loc. cit.), arguing in favour of a *Sarcostemma*, interpreted the *aṃśavaḥ* as the 'Stengelglieder', the internodes of the stems—rather too botanically precise in respect of a Vedic term. From the cylindrical shape of these he derived the sense of the fringes or tassels on a garment, which he considered was implicit in *aṃśupatṭa*. We cannot discuss in detail here the development of the post-Vedic meanings of *aṃśu*; but it is relevant to observe that at that time Renou (loc. cit.) excluded from the *mantras* the common classical sense of 'ray of light'. He added, however, 'Il est possible que cette nouvelle acception ait été déjà présente à l'auteur d'AV. XIII 2 7 qui nous dit du char solaire qu'il est *aṃśumánt* : encore que Henry Hy. Rohitas p. 9 ait probablement raison de maintenir une version ritualisante "chargé de soma". . . . En tout cas la bifurcation de sens s'avère dans un autre passage de l'AV. VIII 1 2, où le poète réclame pour un malade l'assistance de soma *aṃśumánt* : soit à la fois le soma avec les rameaux ou rejets de sa plante, et la lune avec ses rayons'. Possible,

⁷ This was a surprise, at least to me : having taken 'stalk' for granted, I had not previously thought to consult Grassmann for this word.

⁸ i.e., leaving out of account 8.5.26, where *Aṃśu* is a man's name, and 1.100.16, where *sumād-aṃśu* is unexplained, but appears to be part of a horse's harness.

⁹ R. Roth, 'Ueber den Soma', *ZDMG*, xxxv, 1881, 684; L. Renou, 'Les éléments védiques dans le sanskrit classique', *JA*, ccxxxj, juillet-sept. 1939, 341.

yes ; but still speculation for a period as early as the *Atharva-veda*. On the other hand, it is very likely that the meaning of ' ray ' arose—whether in late Vedic or in post-Vedic Sanskrit—from the sun's being *aṁśumant-* 'possessing Soma-plants, the plants called *Aṁśu* ', which would easily lead to a subsequent (mis)understanding as ' possessing rays '.

Like *aṁśu* in relation to Soma in the RV, the Avestan *qsu* is used only of Haoma ; and it would be worth investigating the possibility that here also *qsu* may be the name of the plant itself. (Bartholomae, *AIr. W.*, s.v. *qsav-* : ' Schoss, Zweig der *Haoma*-pflanze '.) In *Yasna* 10.2, for example, it seems possible to understand *qsuṣ* (Parsi Sanskrit version, *pallavān*) as ' the *Aṣu*-plants (belonging to the god Haoma) '. In *Yasna* 9.16 *haomō . . . nāmyqsuṣ*, the Pahlavi version *narm tāk* is followed by the Sanskrit rendering *mṛdu-pallavaḥ*, and by Bartholomae, ' mit biegsamen, zarten Schösslingen '.¹⁰ We might translate as ' Haoma . . . whose *Aṣu*-plants are tender (?) '.

Monier Williams's dictionary, s.v. *aṁśu*, has ' a filament, especially of the Soma plant '. It is amusing to imagine the process of pressing *soma*-juice from the filaments of plants. But the mistake is surely due only to a misunderstanding of the nuance intended by the Petersburg lexicon's ' Faser ', which must be taken here as ' fibre '. This sense cannot be accepted for the RV ; but its genuineness in later Indo-Aryan is well attested : Bengali *āś* (Old Beng. *āsu*) ' fibre of tree or stringy fruit ', and examples from other languages.¹¹ In Classical Sanskrit, a meaning such as ' fibre ' can be seen in Sāyaṇa's comment on RV 10.17.12 *yās te drapsāḥ . . . yās te aṁśūḥ* : commentary, *drapsāḥ rasaḥ . . . yās ca te tvadīyah aṁśuḥ rasād itaraḥ san*, i.e., the *aṁśu* which is Soma's other part in contrast to the juice ; and similarly on verse 13 of the same hymn.

Although we reject ' fibre ' as the precise sense of *aṁśu* in the RV, the later development of such a meaning strongly suggests that the Soma-plant was fibrous or stringy, rather than of the fleshy texture of a mushroom. I am indebted to my wife for the pertinent observation that, if the Soma-plant had been a mushroom, it would be strange that the elaborate Vedic process of pounding out and filtering the juice should have been necessary. Why should the plant not have been simply eaten? There is no particular sanctity in liquid as such : ' cakes ' (*puroḍāś-*, *puroḍāśa-*) are among the sacrificial offerings most frequently mentioned in the ritual texts. Even although dried specimens of the mushroom may have been somewhat tough, even after soaking in water, nowhere in Wasson's ' Exhibits ' is there any mention of the Siberian tribes pounding the fly-agaric to extract juice. On the contrary, in Siberia the plant is regularly eaten or swallowed whole, although occasionally it is used to make a decoction or infusion (pp. 234, 253, 260, etc.), or it is added to soups or sauces (p. 324). But RV 7.26.1 is explicit : *nā sóma índram ásuto mamāda nābrahmāno*

¹⁰ *AIr. W.*, s.v., analysing the compound as *nāmi-qsu-*. Some doubt may still be felt about the sense of **nāmi-*.

¹¹ See R. L. Turner, *Comparative dictionary of the Indo-Aryan languages*, s.v. *aṁśu-*.

maghāvānaṃ sutāsaḥ 'Soma unpressed has never intoxicated Indra, nor the pressed juices unaccompanied by sacred hymns'.

In Indo-Iranian terms, the god Soma acts through the juice to produce *mada*, *maḍa* 'inebriation, intoxication'. Such Western terms, however, have overtones which are out of tune with the reverential awe accorded to Soma. Geldner remarked in the introductory note to his translation of RV 9 (HOS, xxxv, 2), 'Die Wirkung des Somasafts wird im Veda wie im Avesta durch *mad* bezeichnet. Übersetzt man dies mit "berauschen", so ist das fast zu viel gesagt, mit "begeistern" zu wenig'. It is difficult to give an adequate equivalent, but the tenor of the hymns indicates something like 'possession by the divinity', in some way comparable to Greek *ἡ ἐκθουσιασμός*. One inspirational aspect of Soma is clear: more than any other god, he stimulates the poetic creation of the Vedic seers. All the words concerning poetic inspiration appear in connexion with Soma, such as *dhī*, *dhīti*, *mati*, *vip-*, *gir-*, *manīṣā*. The term *kavi* 'poet' is applied to men and to other gods, but very frequently to Soma, who is also *ṛṣi*, *vipra* (9.87.3): for Soma as *kavi*, see especially 9.96.17-18. In contrast to the wider application of *kavi*, it is noteworthy that the derivative *kāvya* 'poetic creation' (normally translated by Renou as 'pouvoir-poétique') is used preponderantly in connexion with Soma and Agni, with whom Soma is so closely associated in the RV. In 3.1.8 the two deities virtually coalesce: *ścótanti dhārvā mādhuṇo ghr̥tāsya vṛṣā yātra vāyrdhé kāvyena*, Renou 'tombent-goutte-à-goutte les coulées du doux (*soma*), du beurre-fondu, là où le mâle (Agni) s'est invigoré grâce au pouvoir-poétique (des hommes)'; but perhaps rather, 'the streams of *madhu* (*soma*) drip, the streams of melted butter, where the Bull (Soma/Agni) has increased by means of his (Soma's) *kāvya*'. In 9.66.19-21, Agni is completely identified with Soma; in 9.96.18 *padavīḥ kavīnām* echoes the sense of 9.62.25 *vācō agriyāḥ*—Soma as 'leader of the Word'.

A *kāvya* is not merely an 'inspired utterance', but often a 'magically potent spell': in 4.35.4 the Ṛbhus divide the cup into four by their 'magic (creative) power', *kāvya*, and are invited in the same verse to press and drink *soma*. Similarly, the two Aśvins came to the aid of Indra by means of their magic skills, their wondrous powers: 10.131.5 ... *aśvīnobhēndrāvāthuh kāvyaīr dāmsānābhīḥ*. (See Geldner's note on the confused syntax of this verse. The general sense, however, is clear.) As a further development, elsewhere the plural *kāvyaṇi*, usually with the adjective *viśvāni*, appears to have acquired virtually the sense of '(all) things created (by the Word)'. For the recurring phrase *abhi viśvāni kāvyā*, Renou has 'pour (atteindre) tous pouvoirs-poétiques' and similar renderings; but it may rather be taken as 'towards all created things'. See especially 9.70.2 and 9.107.23. A strong confirmation is provided by 8.41.5-6, where there is a parallel between the two verses: *yō dhartā bhūvanānām ... sā kavīḥ kāvyā purū ... puṣyati* ('Varuṇa) who is the supporter of the creatures (*bhuvanāni*) ... he, the *kavi*, nourishes the manifold creations (*kāvya*'), and *yāsmīn viśvāni kāvyā cakre nābhīr iva śritā* 'in whom all created things (*kāvya*) are held firm, as the nave is held in the wheel'. In a note

on this passage, Renou wrote of *kāvya* (*EVP*, vii, 30), 'Le mot englobe les arts poétiques, les créations cosmiques et tous savoirs secrets ; *kavi* est du point de vue véd. le créateur, le démiurge spirituel'. He nevertheless retained 'arts-poétiques' in his translation of the same passage. Closely similar is 9.94.3 *pári yāt kavīḥ kāvyaḥ bhārata . . . bhūvanāni viśvā* 'When the *kavi* (Soma) supports on all sides all created things (*kāvya*), all creatures (*bhūvanāni*)'.

Such a development of sense would then be in the direct ancestry of the later philosophical concept of *śabda-brahman*. As early as 1955, Renou put forward a comparable proposal in the very first paragraph of *EVP*, I, 1 : 'Comme on l'a souvent constaté, sur un plan philosophique (ou pré-philosophique, si l'on préfère), un mot tel que *vāc* n'est autre que l'équivalent de logos : c'est le prototype de la notion d'*ātmán-bráhman*, comme le dit G. [Geldner] ad 10.125, hymne adressé précisément à la Parole'. He refrained from making such a sense explicit in his translations of the hymns. Nevertheless, his note on 8.41.5 quoted above indicates that for himself it presumably remained an implicit understanding.

On a more mundane plane, the *soma*-drink was a powerful stimulant for those about to go into battle. In the hymns, it is drunk by the principal gods (9.90.5, and frequently) and above all by Indra, who thus prepares himself for his fights against the enemies of the Āryas and demonic foes, and particularly for his conquest of the demon Vṛtra in order to release the imprisoned waters. In the Avesta the epithet *vərəθraǵā* 'victorious' is used of Haoma (*Yasna* 9.16), while in RV 8.24.6, 10.25.9 Soma is *vṛtrahantama-*, and *vṛtrahan-* in 1.91.5 etc., though in India the epithet is most frequently applied to Indra.¹² We may conjecture that the secular use of *soma* in battle came first in time, although its translation to the divine sphere must quickly have begun to develop in parallel. In the RV, *soma* is predominantly for the gods ; but even here it appears occasionally as a battle-stimulant for human beings : 9.30.3 *ā naḥ śúṣman nṛśáhyam* 'Bring us warrior-might [exaltation of spirit as well as bodily strength] fit to subdue warriors' ; 9.85.2 *asmān samaryé pavamāna codaya . . . jahí sátrūn* 'O Pavamāna, stimulate us in the battle . . . strike down our enemies'.

There is regrettably little further information on the effects of *soma*. The only hymn which appears to give some details is RV 10.119, a soliloquy of one who has drunk the juice.¹³ If, as is usually held, the speaker is a deity or at least a mythological character, we must treat with circumspection any suggestion that this hymn is evidence for hallucinatory effects on human beings, and in any case make allowance for hyperbole. The hymn has often been quoted as

¹² See E. Benveniste and L. Renou, *Vṛtra et Vṛtragna* (Cahiers de la Société Asiatique, III), Paris, 1934; I. Gershevitch, *The Avestan hymn to Mithra*, Cambridge, 1959, p. 158.

¹³ Ancient tradition is unreliable on the myth or ritual for which this hymn was composed, although some connexion with Indra is recognized : for details, see Geldner's introductory note to his translation of the hymn. Modern scholars have tended to identify the speaker as Indra. R. Hauschild argued in favour of Agni : 'Das Selbstlob (*ātmastuti*) des Somaberauschten Gottes Agni (Rgveda x, 119)', in J. Schubert and U. Schneider (ed.), *Asiatica: Festschrift Friedrich Weller*, Leipzig, 1954, 247-88, a view which Renou considered probable (*EVP*, xiv, 39).

evidence that a *soma*-drinker experienced a great expansion in size : verse 12 *ahám asmi mahāmahò 'bhinabhyám údīṣitaḥ* 'Ich bin riesengross, bis zum Gewölk emporgestiegen' (Hauschild). Verse 8 *abhi dyám mahinā bhuvaṁ abhīmām prthivīm mahīm* 'Hinausgewachsen bin ich über den Himmel durch meine Ausdehnungskraft, hinaus über diese grosse Erde' (Hauschild) ; 'J'ai débordé en grandeur le ciel . . .' (Renou). But there seems no reason for attributing this sense to the verb *abhi-bhū-* : rather, 'I have overcome by my greatness [not 'size'] heaven and earth'.

Such a hymn cannot have been composed by a poet under the influence of *soma* : the artifice of its structure excludes this. It is a dramatic monologue, and could easily have been composed by one without personal experience of the original *soma*. It begins with a wish for cattle and horses, a clear echo of the Soma of the earlier hymns who is invoked for booty in war (*vāja*). Two verses then add to this the idea that the *soma* impels the drinker forward like the wind. But verses 4 and 5 then revert to the other aspect, that of poetic inspiration. Only after this does the speaker come to the direct mention of conquest ; and finally—as is fitting for a god—to his setting down the earth where and wheresoever he will, and to his swelling up to fill the space between heaven and earth.

I am prepared to accept 10.119 as a product of poetic imagination. Others are, of course, at liberty to take the hymn as a serious description of the effects of drinking *soma*. Wasson does not cite it as direct evidence for his case, and justifiably so. Yet, if we exclude this hymn, what indisputable evidence is there in the RV that *soma* was 'hallucinogenic' ? There are ample grounds for believing that *soma* was a powerful stimulant, though even here we must bear in mind the possibility of a purely psychological enhancement of its physiological effects. In 8.48.3 *ápāma sōmam amṛtā abhūmāganma jyōtir āvidāma devān*, the poet speaks in exalted terms : 'We have drunk *soma*, we have become immortal, we have gone to the Light (of heaven), we have obtained the gods'. But far more of the Soma-verses are liturgical rather than inspired ; and many other gods are invoked for similar blessings. Exalted language is expected in liturgical utterances, and we can hardly suppose that all of these were drug-induced. The point need not be laboured.

'The roots, leaves, blossoms, seed of Soma : where are they ?' ¹⁴ Wasson considers it odd that 'in a lengthy anthology of lyric poetry written over centuries . . . no poet ever speaks of these conspicuous parts of almost all chlorophyll-bearing plants, not even casually or incidentally'. Short of a conspiracy of silence, 'they were speaking of a plant that had neither seed nor blossom nor leaf nor root ; viz., a mushroom' (p. 18). The RV may in parts be 'lyrical', in a loose sense of the term ; but, to speak with precision, it is not an

¹⁴ Here and in subsequent paragraphs, such italicized headings are those of chapters or sections of Wasson's book. It has not seemed necessary to deal with every point which he raises, and only the arguments which he considers most vital for his case are discussed in detail. I trust, however, that I have passed over nothing of importance.

‘anthology of lyric poetry’. It is a collection of poems, almost all of which are hymns composed for religious rituals. In this religious context, the priests are concerned with the plant itself only at the stage of pressing out the sap : they are entirely preoccupied with the resulting *soma*-juice. On the fly-agaric hypothesis, we might with equal justice inquire, ‘where is the annulus?’—a notable feature of the mature *A. muscaria*. This feature can be seen in plate IX (not very prominently) and in the beautiful water-colour by Charles Poluzzi between pp. 10 and 11 ; but most of the plates show specimens which are too young, or are photographed from an angle which conceals this feature. Apart from the Pavamāna-hymns, other references are to the drinking of *soma* by other gods, or to the mythological aspects of Soma as a deity. When only the product is of religious significance, it does not seem in the least surprising that the priests should not be interested in roots, leaves, blossoms, or seeds, even if the original were a chlorophyll-bearing plant. The same argument is also applicable to Wasson’s view that the epithets and tropes applied to Soma are derived from the botanical characters of *A. muscaria*. Since this view is fundamental to his whole theory, most of his detailed examinations of ‘descriptions’ of the Soma-plant are suspect *ab initio*.

‘*Soma grew in the mountains.*’ This is important for the fly-agaric theory, since the plant grows only in mycorrhizal relationship with the birch and (less frequently) conifers. ‘In Northern Eurasia the birch and conifer grow at sea level. South of the Oxus and in India they are found only at a great height in the mountains, around 8,000 to 16,000 feet’ (p. 23). This would account for the increasing difficulty in obtaining supplies of the plant as the Indo-Aryans penetrated further south. The implicit syllogism is obviously invalid, by reason of the undistributed middle : at this point in the argument, many other plants growing in the mountains may still be considered possible. The twelve RV verses quoted here by Wasson show only that Soma (but plant or god ?) was located on the mountains.

In the Avesta also, Haoma grows on the mountain heights : *Yasna* 10.3, etc., *barəšnuš paiti gairinam*. It seems thus to have been generally accepted that the Soma-plant did grow on mountains. But the facile deduction that it grew exclusively on mountains does not follow logically, and may be contradicted by two passages : *Yasna* 10.17 *vīspe haoma upastaomi yatčit barəšnušva gairinam yatčit ĵafnušva raonam* ‘I praise all the Haomas whether those on the heights of the mountains, whether those in the valleys of the rivers’ ; RV 8.6.28, where the *vīpra* (Soma) is said to have been born *upahvaré girinām samgathé ca nadīnām* ‘in the hidden place [or on the slope ?] of the mountains, and in the confluence of the rivers’.¹⁵ We may note also the name of the river Amśumatī,

¹⁵ In case it might be thought that this last expression is a periphrasis for ‘ocean’, it should be noted that the present verse (*ajāyata*) concerns Soma’s birthplace, whereas the ocean (*samudra*) in connexion with Soma is his ultimate destination, the filtered juice in the sacrificial bowls in the earthly sense, and the ‘heavenly ocean’ in the implied hieratic sense : cf. for example RV I.110.1 ; 9.2.5 ; 9.29.3 ; 9.73.3. On the general question of Soma and the Waters, see Lüders, *Varuṇa*, I, Göttingen, 1951, 225 ff., 272, and J. Gonda, *The meaning of the Sanskrit term dhāman-*,

RV 8.96.13–15, the river ‘abounding in Soma-plants’. This river appears only in a legendary context; but such a name is unlikely to have been used if it had been common knowledge that Soma-plants did not normally grow in river valleys.

On the other side, it could be argued that the *Aṃśumatī* contradicts nature simply because it is legendary; or that river valleys do exist in the mountains higher than 8,000 feet. We might also suggest that the Avestan passage quoted above is not intended literally, but is only a poetic way of saying ‘all Haomas, wherever they may be’. Alternatively, Wasson could reply that such statements might be folk-reminders from a time when the ancestors of the Indo-Iranians knew the birch and the fly-agaric at low altitudes.

But whatever may have been the original plant and its distribution, Soma has become a god; and mountains are natural homes for gods. The Olympian gods of Greece are an obvious example. Viṣṇu is a mountain-dweller (*giriṣit*) in RV 1.154.3—cf. *giriṣu kṣāyaṃ dadhe* of Soma in 9.82.3. In the preceding stanza, 1.154.2, Viṣṇu has the same epithet as Soma, *giriṣṭhā*, while the same line of verse is taken bodily into 10.180.2, where, however, it is applied to Indra. Compare also the reference to Viṣṇu and the Maruts in connexion with the mountains in 5.87.1. In 8.7.1. the Maruts are lords in the mountains (*vī párvateṣu rājatha*), in 8.94.12 they are *giriṣṭhā*-, and in 5.57.8 they are dwellers in the high mountains, *bṛhadgirayah*. Indra is *parvateṣṭhā* in 6.22.2, and is with Viṣṇu on the mountains in 1.155.1 *sānuni párvatānām*. For later Hinduism, one need only mention Śiva, Pārvatī, and Kubera as residents of the Himālaya.

‘*The Two Forms of Soma*’ (p. 25). ‘I now come to a crucial argument in my case. The fly-agaric is unique among the psychotropic plants in one of its properties: it is an inebriant in *Two Forms*. *First Form*: Taken directly . . . by eating the raw mushroom, or by drinking its juice *Second Form*: Taken in the urine of the person who has ingested the fly-agaric in the *First Form*.’ Numerous reports of this phenomenon are cited from the writings of European visitors to Siberia (p. 25, and ‘Exhibits’ *passim*), and although the biochemistry involved still awaits scientific investigation, the use of urine in this way is so widely attested for the fly-agaric in Siberia that we can hardly deny it. Wasson believes that he has found evidence that Soma also had these ‘Two Forms’ in the RV. I am completely unconvinced that he has established this for Soma; but since for him it appears to be the very corner-stone of his case for his identification of Soma with the fly-agaric, his discussion of the point must be dealt with in some detail.

In brief, Wasson’s evidence consists of only two verses from the RV, verses which have nothing in common except that they occur in Soma-hymns: 9.66.2 appears to mention ‘two forms’ of Soma (but gives not even a hint that one of the ‘forms’ might be urine); while 9.74.4 apparently states that the priests urinate the *soma* (but gives no indication that there was any question of

Amsterdam, 1967, 50. The stanza 8.6.28 was thought by Sāyaṇa to refer to Indra; but the view of the commentary on the same verse in VS 26.15, referring it to Soma, is certainly right: cf. *indubhih* at the end of 8.6.27, and Geldner’s note on 8.6.28.

drinking the urine). Apart from doubts about the interpretation of these two verses, which we shall discuss below, it seems to me incredibly weak as an argument to link these two unconnected passages, and to proceed to the deduction that urine was Soma's 'Second Form'.

9.66.2 *tābhyām víśvasya rājasi yé pavamāna dhāmanī*
 pratīcī soma tasthātuh.

Renou translates: 'Avec ces deux formes, (la pure et la mélangée), qui se tiennent face (à nous), ô *soma*, tu règnes sur toutes choses, ô Pavamāna'. It is unfortunate that Renou, following Geldner, should have translated *dhāmanī* here as 'deux formes'. Had he chosen some other rendering, Wasson might not have fallen into this particular trap. In other places, Renou gives for *dhāman* expressions such as 'institution', 'structures', 'positions', '(séjour) institution(nel)'; and it is not always clear to me what his intention was.

The word *dhāman* has been given the most diverse senses by ancient and modern writers. A recent monograph by Gonda¹⁶ presents a good case for understanding the *dhāman* of a Vedic god as 'to a certain extent ... a "location" of a numen, of divine power, of a deity, i.e. not only or merely a "holder" or "receptacle" of divine power, a place, being or phenomenon in which a divinity sets or places itself, functions or manifests itself, or displays its power, or where its "presence" is experienced, but also a particular way of presenting or revealing itself, of locating or "projecting" a mode of its nature and essence, a hypostasis or refraction in which it is believed to be active'.¹⁷ Even if some scholars do not accept unmodified all of Gonda's interpretations of individual passages, this general sense appears to be satisfactory in the RV for the greater part.

It is true that such an understanding of *dhāmanī* in the present verse does not by itself contradict Wasson's idea: it could be argued that the *soma*-juice and the urine were 'two places of manifestation' of the god Soma. Gonda himself compares this verse with 9.68.6, where that *rūpa* (where the sense is indeed 'form') of Soma which was brought from heaven by the mythical falcon is juxtaposed in the same verse with the clarified juice; and he suggests that the *dhāmanī* of 9.66.2 are in fact these two forms. In this, he is following approximately Sāyaṇa's alternative interpretation of the *dhāmanī*, namely, the *aṁśu* (the plant before or during the pressing) as the one, and the pressed juice as the other.

The real stumbling-block is the fact that this verse is the only place where the dual of *dhāman*- occurs, not only of Soma, but in the whole of the RV. The word is frequent in relation to many deities besides Soma, and both the singular and plural are also frequent for Soma himself. In 9.96.18-19 Soma's third and fourth *dhāmans* are mentioned, the first and second being implicitly given in verse 17 of the same hymn—possibly though not certainly the *dhāmanī* of

¹⁶ J. Gonda, *The meaning of the Sanskrit term dhāman-*: see especially pp. 44, 47 ff. for the various *dhāmans* of Soma.

¹⁷ op. cit., 19.

9.66.2. But Soma has also numerous other *dhāmans* : for example, 1.91.4 *yá te dhāmāni divi yá prthivyām yá párvateṣv ósadhīṣv apsú : tébhīr no víśvaiḥ . . .* ‘ With all these *dhāmans* of yours, (O Soma,) those in heaven, those on earth, those on the mountains, in the plants, in the waters . . . ’ ; cf. also 1.91.19. If Wasson’s ‘ *Two Forms* ’ had had any real place in Vedic religion, it is inconceivable that they should have been mentioned once, and once only. And, to put it mildly, the constant and continual mention of all these other ‘ forms ’ would have been very confusing.

Although Wasson does not seem to have realized the complete isolation of the dual of this word, he is of course well aware of the distinction between dual and plural in Vedic and Sanskrit grammar ; and he quotes also 9.66.3 and 5, with *dhāmāni* and *dhāmabhiḥ* respectively. He adds in a footnote (p. 26), ‘ In IX 66² the dual number is used speaking of the *Two Forms*. This is natural as the poet faces two vessels containing, one the juice of Soma presumably mixed with milk, *etc.*, the other Soma urine. In verses 3 and 5 he speaks of all Soma’s forms, the celestial, the plant, the juice, the Soma urine, and therefore uses the plural ’. It is not unreasonable to suggest, in contrast to Sāyaṇa’s and Gonda’s view cited above, that the dual in verse 2 may refer to the two vessels (*camū*) which receive the filtered *soma*-juice. These are well known, and often appear in the hymns. But it is a leap in the dark to suggest further that one of these vessels contains ‘ Soma urine ’. He himself complains, and with some justice, that ‘ the Vedic commentators,¹⁸ knowing nothing of the fly-agaric, have reached a consensus that the *First Form* is the simple juice of the Soma plant, and the *Second Form* is the juice after it has been mixed with water and with milk or curds ’. This explanation, he continues, ‘ is unsatisfactory because it flies in the face of the ṚgVeda text ’. So also, unfortunately, does his own explanation, since 9.86.47 explicitly states that the juice in both vessels was mixed with milk : *yád góbhīr indo camvòḥ samajyáse* ‘ when, O *soma*-juice, you are anointed with milk in the two vessels ’. Further, 9.96.20 *vīṣeva . . . kánīkradac camvòḥ á viveṣa* ‘ like a bull bellowing mightily (the *soma*-juice) has entered the two vessels ’. Soma’s bull-roar is the regular figurative expression for the noise of the pounding-stones on the two pressing-boards (*adhiṣavaṇa-phalake*). Taken in conjunction, therefore, these last two verses show that the juice enters *both* vessels at the time of pressing, and that in *both* the juice was mixed with milk or curds. There is no place left for ‘ Soma urine ’.

The other verse upon which Wasson relies to establish the presence of ‘ Soma urine ’ in the RV is 9.74.4 :

*ātmanván nábho duhyate ghr̥tām páya
ṛtásya nábhīr am̐tam ví jāyate :
samīcīnāḥ sudānavāḥ pr̥ṇanti tām
náro hitām áva mehanti péravaḥ.*

¹⁸ Here he appears to mean some modern Vedic scholars, since he refers only to A. A. Macdonell, *Vedic mythology*, Strassburg, 1897, 82, 106 ; and see also Renou’s translation quoted above, where ‘ la pure et la mélangée ’ seems to be wrong. Sāyaṇa knows nothing of any such interpretation.

This he translates (p. 29), following Renou : ‘ Soma, storm cloud imbued with life, is milked of ghee, milk. Navel of the Way, Immortal Principle, he sprang into life in the far distance. Acting in concert, those charged with the Office, richly gifted, do full honor to Soma. The swollen men piss the flowing [Soma] ’. (In passing, note that ‘ richly gifted ’ is a misunderstanding of Renou’s ‘ aux beaux dons ’ : *sudānu-* is ‘ richly giving ’—though possibly the sense of ‘ moisture ’ is preferable here : Grassmann, ‘ tropfenreich, schön träufelnd ’.)

Geldner translates the second half of the verse as : ‘ Vereint stellen ihn die Gabenschönen zufrieden ; den zur Eile Getriebenen pissen die schwellenden Männer herab ’ ; and he adds the laconic footnote, ‘ Zunächst die Priester, zugleich aber Anspielung auf die Marut, worüber 2,34,13 zu vergleichen ’. In the verse referred to, the relevant phrase is *nimégghamānā átyena*, i.e., the Maruts (Rudras) pouring down rain, figuratively considered as the urine of their heavenly horse(s).

It has been suggested that two distinct roots are involved : (a) with IE **-gh-*, whence Ved. *meghá-*, Av. *maēya-* ‘ cloud ’ ; Ved. *mih-* ‘ mist, drizzle, rain ’ ; and (b) with IE **-ǵh-*, whence Ved. *méhati*, Av. *maēzaiti* ‘ urinate ’ ; Ved. *mih-*, ptc. *mūḍha-* ; *meha-* ‘ urine ’ ; *medhra-* ‘ penis ’. For further details, see J. Pokorny, *Indogermanisches etymologisches Wörterbuch*, 712–13 ; M. Mayrhofer, *Kurzgefasstes etymologisches Wörterbuch des Altindischen*, s.vv. *megháh*, *méhati* ; H. W. Bailey, *Khotanese texts*, VI, 248, s.v. *biysma*. Since, in the normal development of Indian phonology, the two roots would have collided in the *mih-* forms in Vedic, it is not unexpected that *nimégghamāna-* ‘ raining down ’ should be thought of by the poet as suggesting the urine of a heavenly horse ; and in 1.64.6, quoted below, there would be a pun on the two senses in the word *mihé*. Wasson writes (p. 30), ‘ The blessings of the fertilizing rain are likened to a shower of urine. . . . Urine is normally something to cast away and turn from, second in this respect only to excrement. In the Vedic poets the values are reversed and urine is an ennobling metaphor to describe the rain. The values are reversed, I suggest, because the poets in Vedic India were thinking of urine as the Divine Inebriant, the bearer of *amṛta* ’. I suggest, rather, that an explanation may be the coalescence of two originally distinct words *mih-* in Vedic, and the consequent conflation of other derivatives of the two roots in the minds of the priests, coupled with the obvious fact that ‘ to urinate ’ would, semantically, be a very easy metaphor for ‘ to pour down rain ’. However, such an explanation may well be beside the point, and it would be more economical to assume alternative enlargements in a single IE root. The root *muh-* appears to behave similarly : with **-gh-*, Av. *ašēmaoya* ‘ Irrlehrer ’, and Sk. *mogha-*, *mugdha-* ; with **-ǵh-*, Khot. *māysamḍai* ‘ foolish ’, and Sk. *mūḍha-*. (See H. W. Bailey, *Khotanese texts*, IV, 79.) For the root *mih-*, I. Gershevitch has shown (‘ Etymological notes on Persian ’, in *Dr. J. M. Unvala memorial volume*, Bombay, 1964, 89 ff.) that Modern Persian *mih* ‘ mist, fog ’, contrasting with *mēy* ‘ cloud ’, could represent an OPers. **midam*, Av. **mizəm*, and that Middle Iranian attests related forms with *-z-* where urine is not involved.

Geldner's conjecture that 9.74.4 refers in the first place to the priests pissing in the ritual, though not impossible, seems highly improbable. On the other hand, for reasons given below, the presence of the Maruts in the verse is beyond dispute.

Renou's complete translation of the same half-verse is: '(Agissant) de conserve, (les officiants célestes) aux beaux dons comblent le (*soma*); les (Marut,) seigneurs à la vessie-pleine, compissent (le *soma*) mis-en-branle'. We cannot discuss here the much-disputed meaning of the word *péru-*, *perú-*; ¹⁹ but I should prefer to see in this verse the sense accepted by Renou for *péru-* in his translation of 10.36.8 (*EVP*, v, 52), 'fertilisant', and to translate the last phrase as 'the heroes (the Maruts), fertilizing (the earth), pour down as rain the impelled (*soma*)'. Observe in particular the use of the preverb *áva*, which occurs with *mih-* only here in the RV.

Wasson appears to have thought that Renou was having recourse to a rather far-fetched expedient in an otherwise incomprehensible verse: 'But to give meaning to the sentence he [Renou] introduced the gods of rain, the Maruts. Certainly there are precedents for the clouds' "urinating" rain. But in this verse and at this point in the hymns the Maruts are out of place. From ix 68 to 109 there are 24 other citations of *nṛ* in the plural (men) and in every instance they are the officiants at the sacrifice. So are they in 74⁴' (p. 30).

Neither objection is valid. Granted that in the hymns in question the *narah* are normally the priests, this does not exclude the possibility of a double sense in this verse. In the RV as a whole, the Maruts are called *narah* frequently, probably more often than any other group of deities. They appear not seldom in the Pavamāna-hymns; and in 9.66.26 Soma has them as his 'troop' (*marúḍganaḥ*), while in 9.107.25 the *somas* are 'accompanied by the Maruts' (*pávamānāḥ . . . marútvantaḥ*). Here, then, it is possible to interpret the last line of the verse to mean that the officiants (*narah*) pour down (*áva mehanti*) the streams of *soma* into the receiving vessels, just as the Maruts (*narah*) pour down rain (*áva mehanti*) from heaven. In the case of the priests, such a metaphorical understanding of *áva mehanti* would be hardly more far-fetched than the frequent use of *duh-* 'to milk (the juice from the Soma-plants)'.

The introduction of the Maruts here is no mere guess on Renou's part. In his note on the verse under discussion he remarks that *mih-* 'to urinate' is hardly attested at all in the RV except in the figurative sense of 'rain'; and he compares 1.64.6, where the Maruts are named :

pínvanty apó marútaḥ sudānavah
páyo ghr̥tāvad vidáthesv ābhívaḥ :
átyam ná mihé ví nayanti vājínam
útsam duhanti standáyantam áksitam.

'The Maruts of good wetness (or good gifts?) cause the waters to swell, the ghee-possessing milk (liquid), efficacious in the sacrificial rituals. They lead

¹⁹ For bibliographical details of earlier discussions of this rare word, see M. Mayrhofer, *Kurzgefasstes etymologisches Wörterbuch des Altindischen*, s.v.

aside their booty-winning steed, like a racehorse, to urinate (rain); they milk the thundering, imperishable water-spring.'

Observe that 9.74 is much concerned with rain in other verses also: verse 1 *divó rétasā . . . payovṛdhā* 'the semen of heaven (rain) which increases with milk'; 3 *īše yó vṛṣṭēḥ . . . apām netā* '(Soma) who rules over the rain . . . the leader of the waters'; 5 *ārāvīd amśūḥ śacamāna ūrmiṇā devāvyaṃ mānuṣe pinvati tvācam: dādhāti gārbham āditer upāstha ā* 'The Soma-plant has roared, accompanied by the wave (of the waters), for man it swells up its skin which invites the gods: it places the seed in the lap of Aditi'. Here *tvac-* has the double sense of the outer covering of the plant, and the heavenly leather water-bottle from which the gods pour rain: cf. 1.79.2 *pātanti miha stanáyanty abhrā* 'the rains fall, the clouds thunder', 3 *tvācam prñcanti* 'they fill(?) the leather water-bottle'; 1.129.3 *pinvasi tvācam* 'you (Indra) swell up the leather water-bottle'; also 5.83.6 *divó no vṛṣṭim maruto rarīdhvam prā pinvata vṛṣṇo áśvasya dhārāḥ*, and 7 *dṛtim*.

If we now compare the vocabulary of 9.74 with that of 1.64.6, the agreements are seen to be far too many to be accidental: *pinvanti apó—pinvati tvācam; sudānavah, páyah, and ghṛtá-* in both; *duhanti—duhyate; mihé—áva mehanti; stanáyantam—ārāvit; ákṣitam—amṛtam*. The 'imperishable water-spring' is by implication Soma: cf. 1.154.6 *vīṣṇoḥ padé paramé mádhva útsaḥ* 'in the highest footstep of Viṣṇu there is a spring of *madhu* (i.e. *soma*)'.

This is beyond mere coincidence. Renou did not 'introduce' the Maruts into 9.74.4 as an expedient 'to give meaning to the sentence': his note referring to 1.64.6 shows that he was aware that the terminology of the verse compelled the interpretation which he gave. There is no need to see 'Soma urine' in Wasson's sense, therefore, in 9.74.4 either: only the *soma*-juice itself flowing into the sacrificial vessels, poetically conceived as the pouring down of fertilizing rain from heaven.

As if it were confirmation that fly-agaric urine was a feature of Indo-Iranian religion (and hence pre-Vedic), Wasson quotes also (p. 32) an isolated phrase from one of the Gāthās of Zarathushtra, *Yasna* 48.10 *kadā aṣṇ mūθrām ahyā madahyā* 'When will you (O Mazdāh) smite down the urine of this intoxication?' (not, as in the translation quoted, 'this urine of drunkenness'). It seems certain that Zarathushtra here is castigating the Haoma-ritual; but there is no reason to see in the word *mūθra* anything more than a strong term of abuse.

It is convenient to recall here that the Avesta knows also *hurā* (discussed at the beginning of this article) and *maḍu-*. The latter is given by Bartholomae ' (Wein usw.) Beerenwein '. While this may possibly be anachronistic for the earlier period, there is no doubt that the word meant 'wine' at a later time: Sogd. *mḍw, mwḍ-*, Khot. *mau*; ²⁰ also Central Asian Prakrit *masu* 'wine'. The etymological connexions are well known: Classical Sanskrit *madhu* 'honey',

²⁰ I. Gershevitch, *Grammar of Manichean Sogdian*, § 408; H. W. Bailey, *Khotanese texts*, VI, 284.

where the sense is confirmed by *madhukara* 'bee', and by Tibetan *sbran-rtsi*, Chinese 蜜 'honey' in translating *madhu* in Buddhist texts; Greek μέθυ 'wine' (?); English *mead*.²¹ The dictionaries, however, recognize the sense of 'intoxicating drink' in Classical Sanskrit also: *madhumatta* and other compounds. Compare also Pāṇini 4.2.99 (mentioned by Dr. O'Flaherty, p. 115, n.), where the *Kāśikā* commentary, giving as examples *kāpiśāyanam madhu* and *kāpiśāyanī drākṣā*, appears to suggest a connexion between the grapes of Kāpiśī and the *madhu* of the same region: *madhu* then possibly meaning here 'grape-wine'. In the RV the word *madhu* is very frequently linked with *soma*, and often by itself denotes or implies *soma*. In 1.117.6 and 1.116.7, *madhu* and *surā* appear in parallel contexts (see above, p. 331). It is thus not improbable that in the RV *madhu* does not refer to the 'sweetness' of *soma*, but rather that it conveys the sense of 'soma, the (divine) intoxicant'. In the circumstances, it is slightly odd to find Wasson writing (p. 16), 'Honey, *mádhu*, is mentioned frequently in the R̥gVeda but mead never'. When the words are etymologically the same, how can one draw such a distinction? By these remarks I do not mean to revive the absurd theory that *soma* was mead; but it is not impossible that the Vedic Indians conceived the pressed juice as a sort of 'mead', in respect of its exhilarating properties.

' "Hári" and Red' (p. 36). 'Hári is the most common of the colour epithets for Soma in the R̥gVeda. Numerically it far exceeds all the other colour words put together and rivals the epithet "bull" that the poets never tire of applying to Soma. The word *hári* is cognate with *híraṇya* (golden) in Sanskrit and with *χόλος* (gall) and *χλωρός* (yellow) in Greek, and ultimately with the English "gall" and "yellow". *Hári* is the precise adjective that one would wish to employ in Vedic to describe the fly-agaric. *Hári* is not only a colour word: the intensity of the colour is also expressed by it. It is dazzling, brilliant, lustrous, resplendent, flaming. [On what evidence, one wonders, are the last two sentences based?] In colour it seems to have run from red to light yellow.'

Much of this is acceptable, but the slanting of the linguistic evidence is breathtaking. The word *hári* 'golden' is precisely not the adjective which one would wish to apply to the flaming red fly-agaric; and I have been unable to find any evidence that any shade of red is included in the colour-range denoted by *hári*. The Greek *χλωρός* can be used of honey, but its normal sense is 'greenish-yellow, the colour of young grass, pale green'. Compare also the modern term 'chlorophyll' itself. Wasson adds a footnote, 'Occasionally in later times *hári* came to include green among its meanings, but this usage seems not to be R̥gVedic, except possibly in the late hymns that we exclude from consideration'. It may well be that the sense of 'green' does not occur in the RV, but it is not the case that this sense is a post-Vedic development. It is attested in Iranian, where Avestan *zairi-gaona-* is used of Haoma, 'golden-coloured', but also of *urvarā-* 'plants', where it must mean 'green'. Sogdian *zrywn* is 'green', and

²¹ For other cognates, J. Pokorny, *Indogermanisches etymologisches Wörterbuch*, 707.

‘vegetable’.²² In Khotanese, the derivative of the same word, *ysarūna*- renders Skt. *harita*- (usually green, of grass, or greenish-yellow), and ‘green’ (of beans); and ‘golden’, is well attested, *ysaraqūna* translating *suvarṇa-varṇa*, *ysarraqūnā* where the Tib. has *gser-gyi kha-dog-can*, both ‘of golden colour’.²³ From M. Boyce I have Parthian *zrgwng*, used as an adjective for a juniper tree; and *hwzrgwn* (also in Manichaean Middle Persian), describing a garden, a hill upon which sheep graze, and trees. Modern Persian has *zard* ‘yellow’, *zarrīn* ‘golden’. In later Sanskrit, the meaning ‘yellow’ predominates, with doubtless a tinge of brown or tawny when *hari* is used of lions or monkeys. For Buddhist Sanskrit, the *Mahāvvyutpatti* has *harita*, Tib. *ljan-khu*, Ch. 綠 ‘green’; and *hari*, Tib. *ser-skyā* ‘light yellow’, Ch. 黃 ‘yellow’. I. Gershevitch has drawn my attention to Parsi Sanskrit *lohita*, *pāṭala*, used to translate Avestan *zairita*; but this is much too late to be relevant, and doubtless indicates only imprecision on the part of the Parsi translator.

In addition to Indo-Iranian, the cognates of *hari* are spread in great profusion throughout all the main groups of the Indo-European languages. There are a very few isolated examples where the colour-range seems to have been extended beyond the green, so that ‘blue’ or ‘grey’ may occur in some languages. But overwhelmingly, the other Indo-European languages have cognates denoting ‘golden, yellow, yellowish-green, green’, but nowhere at all any which suggest ‘red’.²⁴

Not only from Indo-Iranian, then, but from Indo-European as a whole, we have incontrovertible evidence that *hari* belongs to a group of words which in the parent language covered that part of the spectrum which runs from yellow into the green. There is no evidence that its range extended even into the orange; and red is absolutely excluded.

Thus the majority of Wasson’s colour plates of the pure red fly-agaric are irrelevant to all points which he intends to illustrate by them in relation to the word *hari*. Plate IV, for example, shows the round red ball of a fly-agaric which has lost its white specks, and is labelled ‘The sun’. Under appropriate climatic conditions, the sun may look like this at dawn or sunset: but this is not *hari*. On the other hand, it is entirely appropriate that the horses of the sun should be *hari*, ‘golden’.

It would be unfair to suggest that this mistake was an intentional bending of the evidence, although one may suspect some degree of special pleading due to the fact that the author had already made up his mind that the fly-agaric was Soma. Most probably he was unconsciously simply taking a step beyond the mistranslations of Bhawe, who, besides ‘yellow one’, renders *hari* in different hymns as ‘reddish-brown’, ‘tawny-brown’, ‘golden-brown’, ‘yellow-brown’—but not ‘red’ or ‘scarlet’.

It is also possible that Wasson was unduly influenced by Renou’s regular

²² Gershevitch, op. cit., § 1113.

²³ H. W. Bailey, *Khotanese texts*, VI, 289, q.v. for more detailed information.

²⁴ Pokorny, op. cit., 429 ff.

rendering of *hari* in the Soma-hymns by 'alezan'. He would have been better advised to have followed Geldner here. The German 'falb' may sometimes be used as a colour-adjective, but 'der Falbe' is almost inevitably a horse, a 'creamy-yellow' horse. For Soma and the horses of the sun, Geldner almost always translated *hari* as 'der Falbe'. Renou apparently intended to follow Geldner's view that the *hari* Soma was regularly a horse, since in French 'un alezan' is always and exclusively a horse: the big French lexicons are unanimous in this. Unfortunately, the colour is as misleading as the earlier English rendering 'bay horse'; for 'alezan' is a horse of a reddish-brown colour, virtually 'chestnut horse'.²⁵ At least one French dictionary, however, takes 'alezan' to be a yellow horse.²⁶ I cannot tell whether this is a mistake, or whether some speakers of French have only a vague idea of the meaning of the term. Certainly, as a speaker of English with no acquaintance with horsemanship, I myself have no clear knowledge of the meaning of many terms connected with horses. Renou may have thought that 'alezan' was a good translation of 'Falbe'; or he may have intended merely to make it clear that Soma as *hari* was a horse, without considering the colour of the horse relevant for his purpose. For the fly-agaric hypothesis, however, the colour is highly relevant: *hari* is 'golden', and when Soma is thought of as a horse, it is a 'golden horse', not a red one.

Wasson also quotes other colour-adjectives applied to Soma (p. 37): *vṛṣā śónaḥ* ('the red bull'); and *aruṇa*, *aruṣa*, *babhru*. We have here a reasonable mythological situation: when Soma is associated with the sun, or occasionally identified with the sun (*sūra*-), he is 'golden', or a 'golden horse'; when associated with Indra, the thundering rain-giver, Soma is the bellowing bull, and appropriately has colour-terms tending towards the red. In mythological thought, there is no contradiction in Soma's appearing in both roles simultaneously: 9.8.6 *arusó háriḥ*, Renou 'le (dieu) fauve, l'alezan', Geldner 'der rötliche Falbe'; but perhaps rather 'the red (bull), the golden horse'. In 9.66.26, Soma is *hariścandra*:- Renou 'brillant (comme) l'or', perhaps following Geldner, 'der Goldschimmernde'. But in 3.44, where there is much verbal play on *hari*, *harita*-, etc., Renou, with one exception, takes the sense of 'golden'; but Indra's epithet *haryaśva* is translated in verse 2 as 'aux chevaux alezans' (Geldner, 'goldrossiger Indra'), and in verse 4 as 'aux chevaux d'or' (Geldner, 'der Goldrossige'). Possibly this is a hint that Renou intended 'golden' throughout.

²⁵ *Dict. historique de la langue française* (Académie Française), 'De couleur fauve, tirant sur le roux. Il ne se dit qu'en parlant de chevaux'; Littré, 'le corps est recouvert de poils rouges ou bruns plus ou moins foncés'. Professor W. Simon has kindly confirmed for me the yellowishness of *Falbe*, quoting also Kluge, *Etymologisches Wörterbuch der deutschen Sprache*, and the reddishness of *alezan*: Meyer-Lübke, *Romanisches etymologisches Wörterbuch*, which gives 'braunrotes Pferd'. The French word is borrowed from Spanish (ultimately of Arabic origin), and the Spanish *Larousse* defines *alazán* as a horse with hair more or less *rojo canela* 'cinnamon red'.

²⁶ Hatzfeld and Darmesteter: '(En parlant d'un cheval.) Qui a la robe d'un jaune plus ou moins clair'.

In passing, we may note that Indra is described as *haryaśva* more than twenty times in the RV, the epithet being exclusively his: and the poets may sometimes have felt that part of the sense at least was 'having Soma as his horse'.

On p. 41 Wasson describes vividly how the brilliant red of the fly-agaric fades with the daylight, until in the late dusk or by star or moonlight the mushroom appears to shine with a pale silvery colour. This, he believes, is the phenomenon referred to in 9.97.9d *dīvā hárir dádṛṣe náktam ṛjrah* 'le jour il apparaît couleur-d'alezan, la nuit, blanc d'argent' (Renou). Wasson translates, 'By day he [Soma] appears *hári* [colour of fire], by night, silvery white'. This is illustrated in plate VIII by two charming photographs, one of a group of red fly-agarics by daylight, the other showing the same group photographed in near-darkness; and the latter are indeed silvery white. Unfortunately, this will not do. Enough has already been said to show that *hari* is not red. And it is not entirely frivolous to remark that there are many colours of fire: RV 10.20.9 lists seven of these, *krṣṇa*, *śveta*, *aruṣa*, *bradhna*, *ṛjra*, *śona*, and *hiranya-rūpa*. Here the reddish shades of fire are described as *aruṣa* and *śona*, while *hiranya-rūpa* 'colour of gold' obviously corresponds to *hari*. Except for this verse, all other eleven instances of *ṛjra* in the RV refer, either explicitly or contextually, to horses. The word thus definitely belonged to the vocabulary of horsemanship.

In the first line of 9.97.9, Soma is called *urugāya*: Viṣṇu in his solar aspect crossing the sky. In line 3, *parīṇasām kṛṇute tigṃśāṅgo* 'He of the sharp horns fills out his full extension', we may see the poet fancifully visualizing the horns of Soma the bull as the horns of the waxing moon. But even without the assistance of lines 1 and 3, the last line of the verse is open to one interpretation only: 'by day, he appears as a golden horse, by night as a silvery horse'. Geldner's brief footnote is entirely justified: 'Soma als Sonne und Mond'.

Indra, as already noted, is *haryaśva*: the use of *ṛjra* as a word for a horse-colour is further justified by the appearance in the RV of *ṛjraśva* as the proper name of a man, 'possessor of *ṛjra*-horses'. A man of the same name is mentioned in the Avesta, *ərəzrāspa* (Yast 13.121). As the Vedic shows, the sense 'des Rosse gradaus, gradan gehen', given by Bartholomae for the Avestan name, is wrong. F. Justi (*Iranisches Namenbuch*, 89) renders the name as 'braunrothe Rosse habend'; but, in giving the wrong colour, he is merely following Grassmann.

It should be added that Geldner and Renou are not necessarily right in seeing Soma as a horse in every place where he is called *hari*, even if this idea must often have been in the minds of the composers of the hymns. Soma is admittedly compared with a horse: 9.71.6 *ásvo ná devām ápy eti yajñíyah*; 8.2.2. *ávyo váraviḥ páripūtaḥ ásvo ná niktó nadīṣu*; 9.65.26 *prá śukráso vayojuvo hinvānāso ná śáptayah*; 9.88.2 *átyo ná mṛṣtáh*; also 9.86.26 and 9.109.10. We cannot at present exclude the possibility that 'golden' refers in the first place to the colour of the plant, or, more probably, to the colour of the *soma*-juice. The same uncertainty as between mythological characteristics, the plant, and

the sap applies to *babhru* and other colour-adjectives. This would still leave us with a wide choice of possible plants.

Another term applied to Soma, *āśu-*, is also often taken to mean 'swift (horse)'. Perhaps this too is sometimes right, and *āśu* 'the swift one' can certainly mean 'horse' elsewhere in the RV. For Soma, modern translators fluctuate between 'swift (horse)' and simply 'swift'. If the former was intended for Soma by the composers of the RV, it may seem to be an Indian development, since in the Avesta, *Yāšt* 10.89, the priest Haoma is *āsu.yasna-* 'promptly-sacrificing' (Gershevitch, *The Avestan hymn to Mithra*). Conceivably, the Indo-Iranian use of 'swift' in this context originally referred to the speed with which the *soma*-drug took effect. In contrast to poisoning with the much more deadly *Amanita phalloides*, where the symptoms often do not appear for many hours, in cases of poisoning by *A. muscaria* (mycetismus nervosus) the onset of the symptoms is very rapid, appearing within a few minutes to within two hours after ingestion. Here we have a coincidence which is nicely consistent with Wasson's hypothesis. But again, it is no proof, since many other available plants may produce rapidly acting chemical substances.

'He makes [of milk] his vesture-of-grand-occasion' (plate VII, and p. 40); 'The hide is of bull, the dress of sheep' (plate IX, and p. 41). These two plates are among the most visually persuasive for Wasson's theory. The 'vesture-of-grand-occasion' is Soma's *nirṇij* (Renou, 'robe-d'apparat'). The photographs show the bright red skin of the fly-agaric tufted over with the fragments of the milky white envelope as it breaks up, the 'tufts of snowy wool'. Plate IX illustrates 9.70.7d *gavyāyī tvāg bhavati nirṇig avyāyī*. On this Wasson writes, 'At least some of the poets knew their fly-agaric *in situ*, high in the mountains: could the last phrase in this verse have been written by anyone who did not know it?'. The answer is, yes: the verse could easily have been composed without any knowledge of the fly-agaric, and almost certainly it was so composed.

'The hide is of bull': this refers to the *adhiṣavaṇa-carma*, the bull-hide spread over the pressing-boards (*adhiṣavaṇa-phalake*) on which the plants were pounded. A bull-hide was chosen for this purpose probably partly because it was the most convenient material available, and, perhaps more important, because it was theologically relevant in respect of the thundering bull Indra and the bull Soma. It is not a guess that the bull-hide here is the one used at the pressing-ritual: the point is implicit in the earlier part of the same verse: *ruvāti bhīmō vṛṣabhās . . . ā yōniṃ sōmaḥ sūkṛtaṃ nī ṣḍati* 'The terrible bull roars . . . Soma sits down in his well-fashioned *yoni*'. Soma 'roars' when pounded by the pressing-stones; and his 'birthplace' here, as frequently, is not the mountain-home of the plant, but the place from which, at the pressing, the juice, the *soma*, is born from the plant. In several other verses, the same bull-hide is explicit: 9.65.25 *pāvate . . . hinvānō gōr ādhi tvaci* 'He purifies himself, being impelled on the hide of the bull'; 9.66.29 *eṣā sōmo ādhi tvaci gavāṃ krīḍaty ādribhiḥ* 'This *soma* sports with the pressing-stones on the hide of

the oxen'; 9.79.4 *ádrayas tvā bapsati góṛ ádhi tvací* 'The pressing-stones devour you on the hide of the bull'.

Similarly, 'the dress of sheep' refers to the ritual, not to the plant, Soma's *nirñij* being here the woollen filter. Since a filter was necessary, sheep's wool was a natural choice of material. Again, the fact that the reference is to the pressing-rite is made explicit in another hymn; 9.99.1 *śukrām vayanty ásurāya nirñijam vipām ágre* ' (the fingers of the priests) weave for the Asura (Soma), at the beginning of the sacred hymns, a white festal garment'. In other words, the woollen filter as festal garment was made at the time of pressing; and there is no hint anywhere that the natural plant possessed a garment of wool.

For Wasson's theory, it might be argued that, granted the direct reference in the hymns is to the ritual use of the bull's hide and the woollen filter (or the mixing-milk), nevertheless these materials were chosen precisely because the fly-agaric has a red skin and a garment resembling tufts of wool. But this would assume the point which is to be proved. If and only if we can *first* prove that the Soma-plant was the fly-agaric, then and only then can we suggest that these ritual features were influenced by the characteristics of the natural plant: otherwise we are arguing in a circle. So far as I have been able to discover, the text of the RV never links any of the ritual facts to the features of the living plant.

Although in these verses Soma's *nirñij* is the woollen filter, the term is much more frequently used of milk or curds with which the juice was mixed. The mixing of the juice with milk is mentioned so often in the Pavamāna-hymns that it is superfluous to cite references. Among those passages where the milk is directly called Soma's *nirñij*, a few may be quoted as examples: 9.14.5 *gāḥ kṛvānó ná nirñijam* 'making milk, as it were, his festal garment'; similarly 9.86.26; 9.68.1 *índavo ... barhiṣádo ... usríyā nirñijam dhire* 'The soma-juices ... seated on the ritual-strew ... have donned as their festal garment the (milk of the) cows of dawn'. As everywhere, this garment is put on at the time of pressing: the Soma-horse whinnies, i.e., resounds under the blows of the pressing-stones: 9.95.1 *kánikranti hárir á srjyámānah ... punāndḥ ... kṛṇute nirñijam gāḥ* 'The golden horse whinnies mightily while being released [i.e., the juice being set free from the solid parts of the plant]; being purified ... he makes his festal garment of milk'. With the same sense, 9.97.2 *bhadrā vástrā samanyā vásāno ... camvoḥ pūyámānah* 'Putting on auspicious festive garments ... being purified into the two receiving vessels'. This mixing must have been common Indo-Iranian: *Yasna* 10.12 *haomō gaoma* 'Haoma, milk-possessing'; *Yāst* 10.6 *haomayō gava*.²⁷

The mixing with milk or curds is discussed in some detail by Wasson (pp. 27 ff.). It is therefore surprising that, while quoting in full the Sanskrit text of 9.69.5, he should omit from his translation the vital word *camvoḥ*, thus concealing the fact that the whole verse is descriptive of the mixing with milk in the recipient bowls, after the juice has been pressed:

²⁷ On this, see Gershevitch's note on the verse in question in *The Avestan hymn to Mithra*.

ámṛktena rúsatā vásasā hárir
ámartyo nirñijānāḥ pári vyata :
divás prsthām barhānā nirñije krto-
-pastāraṇaṃ camvòr nabhasmāyam.

Renou's translation is: 'D'un vêtement immarcescible, brillant, l'alezan immortel, paré-de-neuf, s'enveloppe tout autour. Avec puissance il a pris le dos du ciel pour s'en parer, (il a fait du lait) un tapis semblable-à-la-nuée pour les deux vases (sômiques)'. Wasson quotes this opposite plate II, but omitting the words '(il a fait du lait)' — a suppletion, but undoubtedly correct—and, more important, omitting also the phrase 'pour les deux vases (sômiques)'. His own rendering of the second half of the verse (p. 41) is: 'By authority he has taken the back [i.e., the vault] of heaven to clothe himself in, a spread-cloth like to a cloud . . .'. The replacement of *camvoḥ* of the original by three dots hides from the reader who does not know Sanskrit the fact that the garment which is *nabhasmayam* is the milk which 'spreads like a mist' through the *soma*-juice in the vessels. As with the bull's hide and the sheep's wool, it is not made clear that the milk as 'the vesture-of-grand-occasion' is assumed by Soma only in the course of the ritual. The verse does not refer to the living plant.

The word *nirñij-* has usually been taken to be etymologically connected with *nij-* 'to wash', Greek *νίω*, etc.:²⁸ hence, 'a thoroughly washed (garment)'. This would be in keeping with later Indian attitudes, which require a new (*anāhata*) or at least a freshly washed garment for ceremonial occasions. H. W. Bailey²⁹ proposed a different etymology: 'Beside the bases Indo-Iran. *naiṣ-* and *naiš-* a third form *nai-g-* could be expected. This base seems to be contained in Rigvedic *nirñig-* 'garment, covering' which has perhaps too long been connected with *neg-* 'to wash' with difficult conjectural intermediaries'. If this proposal is accepted, it may still be the case that the composers of the RV did connect *nirñij-* with *nij-* 'to wash', by the operation of a 'folk-etymology'. In a number of instances, *nirñij-* has no obvious connexion with washing: 1.25.13 *bíbhṛad drāpīm hiraṇyāyaṃ vāruṇo vasta nirñijam* 'Wearing a garment of gold, Varuṇa has put on a *nirñij*'; also 1.113.14; 10.27.24 *sá pādúr asya nirñijo ná mucyate* 'his foot (?)³⁰ is freed as from a garment'; 5.62.7 *hiraṇyanirñik . . . sthūṇā* 'pillar clad in gold'. But, as we have seen, Soma's *nirñij* is more frequently milk than anything else; and the poets appear to play on the sense of 'washing' in a number of hymns. Thus, 9.71.3 *vṛṣāyáte nábhasā . . . nenikté apsú* 'He (Soma) plays the part of the bull by means of the cloud (semen, as an alternative expression for the mixing-milk), he is washed thoroughly in the waters'; and compare 8.2.2 *ásvo ná níktó nadīṣu*, quoted above. In 9.69.5, *nirñijānāḥ* (Renou, 'paré-de-neuf') cannot of course be thought of as a denominative formed from *nirñij-* 'festal garment', but is rather 'washed down', as a play upon words, with *nirñije* later in the same verse.

²⁸ Pokorny, op. cit., 761.

²⁹ BSOAS, xxiii, 1, 1960, 23-4.

³⁰ See Mayrhofer, op. cit., s.v.

Renou's note here (*EVP*, ix, 78) is insufficiently precise. In 9.82.2, Soma's *nirñij* is *ghṛta*; and Agni is naturally *ghṛtánirñij*- in 3.17.1, 3.27.5, 10.122.2; and so also Apām Napāt as Agni, 2.35.4 *ghṛtánirñig apsú*. Mitra and Varuṇa have a *nirñij* of *ghṛta* in 5.62.4 and 7.64.1, where Renou suggests that *ghṛtásya nirñijaḥ* evokes the idea of rain. The Maruts are *varṣánirñijaḥ* in 3.26.5 and 5.57.4

'The udder and Soma' (p. 43); 'The stalk and Soma' (p. 44). 'The swollen hemisphere of the fly-agaric's cap naturally suggests an udder to the poet.' 'Not only is the Soma plant likened to an udder; the stalk or *aṃśu* (literally a "shoot", a perfect word for the stipe of a mushroom) is likened to a teat.' The direct use of the word *údhaḥ* does occur in connexion with Soma; but the second statement is not precise. All that is said is that the *aṃśu* is milked, and a comparison with a teat can then be only implicit. Perhaps this is no bad thing for Wasson's theory, since if the Vedic poets held both of these conceptions, one might be disposed to smile at their curiously inverted idea of an udder suggested by a mushroom with its stipe. If the suggestion made earlier in this article is accepted, that *aṃśu* is not 'stalk' but the name of the Soma-plant, the stipe would not be relevant. No elaborate discussion is needed. When the *soma*-juice is pressed out of the plant, the verb 'to milk' is a natural metaphor, whence 'udder' follows equally naturally. There is no need to invoke the shape of a mushroom to explain such a metaphorical usage.

In passing: 1.137.3 *aṃśúm duhanty ádribhiḥ sómaṃ duhanty ádribhiḥ* supplies an excellent early example of the double accusative with the verb *duh*-, well known in Pāṇini 1.4.51 *akathitaṃ ca*, illustrated by *gāṃ dogdhi payaḥ*.

'Soma's "head"' (pp. 45-6). The suggestion is made that the 'head' in connexion with Soma refers to the pileus, the cap of the mushroom. One of the examples quoted, 9.68.4 *aṃśúr . . . rákṣate śíraḥ* 'the *aṃśu* protects his head', is mysterious, and the sense behind the verse is not made clearer by the mushroom theory. In his note on the verse, Renou conjectured that the 'head' is 'la portion pure ou céleste du *soma* . . . préservée des tribulations de la portion impure—*riprá* 78, 1—ou terrestre'. In the remaining four examples quoted by Wasson, Soma's head, *mūrdhan*-, or his 'head of heaven' *divó mūrdhā*, is present in the filtered juice. This would exclude any solid part of the plant, mushroom or otherwise.

'The single eye' (plate x and pp. 46-7). The fly-agaric in plate x, labelled 'The single eye', is the same photograph of which plate iv, labelled 'The sun', is an enlarged detail. There is no harm in this, since 'the single eye' is in fact the sun, as the verses quoted here by Wasson confirm, though such confirmation is hardly needed. And if we are not convinced that the mythological connexion between Soma and the sun is due to the fly-agaric, 'the single eye' adds nothing. Without attempting to explore other aspects of Soma's solar features, we need only remark that Soma has become a great god, and great gods naturally have the sun as their eye. It is trite to remark that the sun is the eye of Mitra and Varuṇa: 6.51.1, 7.61.1, 7.63.1, etc.; of Indra, 7.98.6 *yát páśyasi cákṣasā sūryasya* 'when you see with the eye of the sun'; and of the gods in general,

7.76.1 and 7.77.3 *devānām cākṣuḥ*. The sun and moon appear as 'the two immortal eyes of heaven': 1.72.10 *divó . . . akṣī amṛtā*.

'*Mainstay of the sky*' (pp. 47-8). Nine passages are quoted in support, and it cannot be doubted that Soma is described as the 'supporter of the sky', *divó dhartā* and similar expressions. Wasson here writes, 'What poet could conceive of a creeper, a climber, any vine—some species of *Sarcostemma* or *Ephedra*—as "mainstay of the sky", "foundation of the earth"? But the sturdy stanchion with its resplendent capital that is the fly-agaric lends itself well to this poetic conceit'. For my part, I find the idea almost comic that the Vedic poets should have seen in this little mushroom a model of the sky supported by a mighty pillar—unless they were indeed suffering severe hallucinatory effects of *soma*-drinking. The natural explanation here, as before, is that Soma is a great god, and one of the regular functions of the great gods is to prop up the sky, or to prop asunder heaven and earth. This is so familiar that it need be illustrated only by a few out of numerous examples: 6.70.1 *dyāvāprthivī varuṇasya dhārmanā viśkabhūte* 'heaven and earth propped asunder by the ordinance of Varuṇa'; 7.86.1 *vī yās tastāmbha ródasī cid urvī* '(Varuṇa) who propped asunder heaven and earth, wide as they are.'; 8.41.10 *yá skambhēna vī ródasī . . . ádhārayat* '(Varuṇa) who held apart heaven and earth by a pillar'; 3.59.1 *mītró dādhāra prthivīm utā dyām* 'Mitra supports the earth and sky'; 1.154.1 *yó áskabhāyad úttaram sadhástham* '(Viṣṇu) who propped up the assembly-place (of the gods) on high'; 3.5.10 *úd astambhīt samídhā nākam* '(Agni) propped up the firmament by means of the sacrificial kindling-stick'; 2.12.2 *yó dyām ástabhnāt sá janāsa índraḥ* 'he who propped up the sky, O men, is Indra'. What more natural than that Soma should take his place in this august company?

'*The Filters*' (pp. 51 ff.). Wasson cites two verses which mention three filters (pp. 54-5), quoted here with his English translation: 9.73.8 *ṛtasya gopā ná dābhāya sukrátus trí śa pavitrā hṛdy antár ā dadhe* 'The Guardian of the *Ṛtā* [Soma] cannot be deceived, he of the good inspiring force; he carries three filters inside his heart'; 9.97.55 *sám trí pavitrā vītatāny eṣy ánu ékaṃ dhāvasi pūyamānaḥ* 'Thou runnest through the three filters stretched out, thou flowest the length, clarified'. For the second of these, the translation is misleading, depending as it does on Renou, but omitting the suppletions; 'Tu parcours les trois filtres (dé,à) tendus; tu coules le long de (chac)un (d'eux, une fois) clarifié'. As so often, Sāyaṇa is not very helpful: *trīṇi pavitrāṇi agnivāyusūryāt-makāni . . . kiṃ ca pūyamānaḥ tvam ékaṃ avivālakṛtaṃ pavitrāṃ anu dhāvasi*. Agni, Vāyu, and Sūrya here seem to be only a guess. But his view that the actual filter of sheep's wool is different from the other three may be correct, and Renou's '(chac)un' seems unjustified. Thus, 'You unite with the three stretched-out filters; (but) in purifying yourself you run the length of the one (namely, the filter of wool)'. Geldner's note on the verse is partly based on Sāyaṇa: 'Die drei sind die mystischen, im Herzen befindlichen (3,26,8); die eine die wirkliche aus Schafhaaren gemachte'. The three *pavitrās* in 3.26.8 are

apparently those factors in the heart or mind (cf. *hṛdy antār* in 9.73.8) which 'purify the inspired hymn in the heart of the poet': *tribhīḥ pavitrair āpupod dhy ārkām hṛdā matīm jyōtir ānu prajānān* (here of Agni). These 'purifiers of poetic inspiration' (plural, though not specified as three) appear elsewhere: 3.1.5 *krātum punānāḥ kavibhīḥ pavitraiḥ* '(Agni) purifying his inspiration by means of the poets, the purifiers' (or, following Geldner's suggestion, 'by means of the poetic purifiers'); 3.8.5 *punānti dhīrā apāso manīṣā*; 7.85.1 *puniṣe vām arakṣāsam manīṣām sōmam indrāya vāruṇāya jūhvat*. Soma is himself a *kavi*, and it is possible that in 9.73.8 he might have placed 'three filters in his heart'; but in the light of 3.26.8 and the other passages just quoted, a more probable interpretation may be: 'he has placed the three purifiers (of poetic inspiration) in the heart (of the poet)'.

Since multiple applications and nuances of words are common in the RV, this proposal does not contradict the only certain aspect of Wasson's 'first filtre', namely, that the hymns on occasion conceive a 'heavenly filter' for Soma. This, however, is easily explained by the axiomatic sacerdotal assumption of parallelism between ritual and cosmic events. Thus, 9.66.5 *tāva śukrāso arcāyo divās prsthē vi tanvate pavitraṃ soma dhāmabhiḥ*, translated by Wasson (p. 52) as, 'Thy clear rays spread over the back of heaven, the filtre, O Soma, . . .'. Renou, however, was probably on the right track when, in view of *dhāmabhiḥ*, he assumed ellipsis, and translated as '(atteignant) le filtre, ô soma, avec (tes) formes (successives)'. The four *dhāmans* of 9.96.18-19 and similar passages quoted above suggest that the verse condenses into a few words the heavenly manifestations and the *dhāmans* at the actual ceremony.

Wasson's 'first filtre' (p. 52) is that 'where the sun's rays, escorting Soma down from the sky, are caught and held on the fiery back of heaven (= the pileus of the fly-agaric)'. Except for the equation in brackets, this is a possible explanation, although there is no certainty that the filter in question is one of the *trī pavitrā* in the two verses quoted above. The 'second filtre' is easily accepted as the ritual woollen filter.

Wasson's interpretation of his 'third filtre' is more difficult. He assumes that in the rite the parts of Indra and Vāyu are performed by the priests impersonating the gods, as in a ritual drama—hence his quotation-marks. He writes (p. 55), 'Let us assume the fly-agaric surmise is well founded. Then the third filtre becomes clear: the Soma juice that is drunk by "Indra" and "Vāyu" in the course of the liturgy is filtered in their organisms and issues forth as sparkling yellow urine, retaining its inebriating virtue but having been purged of its nauseating properties'.

The first passage quoted in support is 9.70.10, but with the omission of the first *pāda*, which is essential for the meaning. The first half of the stanza is: *hitō nā sāptir abhi vājam arṣéndrasyendo jathāram ā pavasva*. By omitting half of this, Wasson gives us the translation, 'Purify thyself in Indra's stomach, O juice!'. Here he is following Renou, 'clarifie toi dans le ventre d'Indra!'. But this is surely a mistake, and an unfortunate one, since it has led Wasson to

believe that a further purification or filtering takes place within the stomach of Indra. Elsewhere, it is clear that Indra consumes the already filtered juice. The sense here is rather: 'Like a horse impelled towards booty (in battle), flow, O soma-juice, into the belly of Indra: purify yourself'.

The other verse upon which Wasson relies to establish 'Soma urine' in relation to the 'third filtre' is 9.63.7 (p. 56): 'Is not the following verse imbued with new meaning, in the light of my interpretation—the *human waters* being put into movement ?

Clarify thou thyself by that stream by which thou madest the sun to shine,
putting into movement the human waters !

*ayā pavasva dhārayā yāyā sūryam ārocayāḥ
hinvāno mānuṣīr apāḥ*

Interpreted so, the verse is certainly 'imbued with new meaning', but a wrong meaning. There is no reference here to human urine: the contrast is between the heavenly waters in the first part of the verse, and the 'waters of mankind' in the latter part—the rains and the rivers with which Soma and Indra are so closely associated. The same *āpo mānuṣīh* are apostrophized in 6.50.7. Comparable is the contrast between heavenly and earthly races in 7.4.1 *daivvyāni mānuṣā janūṃṣi*.

In all the other verses quoted as illustrations (pp. 56 ff.)—and they are many—the talk is exclusively about the ingestion by Indra of the already filtered soma-juice, which enters into his heart, or his belly or entrails. There is no further filtering within the body of Indra: there is no hint in the RV that Indra ever excreted the soma.

'Tongue of the Way' (p. 58). We are told that the cap of the fly-agaric, 'the full blown red tongue, held the clue to the little mystery' of the phrase *ṛtasya jihvā* in 9.75.2. But Wasson himself remarks that 'the poet continues to apostrophize Soma as the source of eloquence'. There is no mystery here: see the discussion earlier in this article on *kavi* and *kāvya*. It is superfluous to seek a further explanation by showing a photograph of a slightly elongated and twisted fly-agaric (plate XIII).

To conclude this section in lighter vein, I cannot refrain from mentioning *sahāsrabhṛṣṭi-*, which Wasson (p. 52) considers to refer to the thousand studs, i.e., the white patches on the cap of the fly-agaric. He is aware that *bhṛṣṭi* 'is used for the knobs or studs on a cudgel, as on the cudgel of Indra. With his thousand knobs or studs Soma conquers potent fame: so say the hymns in two places'. In fact, the half-verse in question is the same in both hymns, except that in 9.83.5 the verbs are in the second person, in 9.86.40 in the third. The conceit is therefore isolated. It is highly probable that in these two verses in book 9 Soma is for the time being thought of as Indra's weapon:

9.86.40 *rājā pavitraratho vājam āruhat
sahāsrabhṛṣṭir jayati śrávo brhāt*

'As king with the filter as chariot, he has mounted upon the booty of war: as

the thousand-studded weapon (of Indra), he conquers high renown'. This seems satisfactory, since that which is *sahāsrabhr̥ṣṭi* is regularly the thunderbolt (*vajra*) or war-club (*vadhā*) of Indra, fashioned for him by Tvāṣṭar, and used by him (admittedly with somic inspiration) in his battle against Vṛtra: 1.80.12; 1.85.9; 5.34.2; 6.17.10; also 1.52.15 *bhr̥ṣṭimātā vadhēna*. How charming is the idea of the tiny fly-agaric as the mighty weapon of the great god Indra!

As one who has no specialist qualifications in chemistry or pharmacology, I can mention only very briefly the problems raised by Wasson's theory for human physiology. In dealing with the use of the fly-agaric in Siberia, Wasson quotes frankly many sources which mention the emetic properties of *Amanita muscaria*. Here we can only conjecture that repeated small doses might acclimatize the subject, and result in a tolerance to the nausea; but obviously scientific investigation is necessary. Even more serious for the Soma theory is the repeated mention in the 'Exhibits' of the coma induced by the fly-agaric: see for example p. 279 ('an ecstatic stupor'); p. 315 ('transports himself into a state of unconsciousness'); p. 248 mentions vomiting and convulsions. Especially disturbing is p. 306, where 'in a stupor from three sun-dried agarics, our Hero is unable to respond to the call to arms. But time passes and the urgency grows, and when the messengers press their appeal to throw off his stupor he finally calls for his arms'. Wasson does not make it at all clear in what way this statement differs in respect of the two versions of the same Vogul tale on pp. 303 and 306. Even with this uncertainty, the coma caused by the fly-agaric is too widely attested to be ignored. Here, it would seem, is a plant whose effects are totally unsuitable to stimulate Indra and human warriors for battle. The other reports that the fly-agaric enables men to carry out great feats of strength, and produces increased strength and agility (pp. 159, 240, 273-4, etc.) are hardly compensation for the disadvantages of stupor and unconsciousness.

Wasson (p. 61, n.) refers to, but does not quote, an article by Bowden, Drysdale, and Mogey.³¹ This article, not unexpectedly, dealt with the effects of the plant on flies. From it the authors extracted muscarine, acetylcholine, and a carboxylic acid which they provisionally identified with the ibotenic acid³² earlier extracted from *Amanita muscaria*, *A. strobiliformis*, and *A. pantherina* by Takemoto, Nakajima and Yokobe.³³ It would appear, however, that it was this last-mentioned constituent which produced a temporary state of unconsciousness in flies. Since it is most probable that the muscarine is the chief nauseating agent in *A. muscaria*, the prospects for this line of pharmacological research are

³¹ K. Bowden, A. C. Drysdale, and G. A. Mogey, 'Constituents of *Amanita muscaria*', *Nature*, CCVI, 4991, 1965, 1359-60.

³² Named from Jap. *ibo-tengu-take* (presumably 'verrucose *Amanita*') = *A. strobiliformis*. *A. muscaria* is *beni-tengu-take* 'red *Amanita*'. The use of *tengu-take* 'goblin-mushroom' for *Amanita* may be of interest for vernacular fungal terminology. [The discussion of 'muscarine-effects' here and on p. 361 may now require modification: see Addendum to footnote on p. 362.]

³³ *Journal of the Pharmaceutical Society of Japan*, LXXXIV, 1964, 1232.

not very promising for Wasson's Soma theory. It is nevertheless encouraging that Wasson reports (p. 202) that work on the chemistry and pharmacology of the fly-agaric is in progress in Zürich and in Japan.

One perplexing factor in the situation is that the modern Zoroastrians identify the Haoma-plant as a species of *Ephedra*.³⁴ This naturally does not prove that the latter was the original plant. So far as I know, it has never been used as a substitute for Soma, and all the references to it in modern discussions of the Indian Soma appear to have been derived from Iranian information (see Dr. O'Flaherty's chapter in Wasson's book). Even if an *Ephedra* might have been the original Soma—and I must make it clear that I am not suggesting that this was so—it would probably be impossible to identify the species.³⁵ The alkaloid ephedrine (similar in its physiological action to adrenaline—in the United States called epinephrine) was isolated by Yamanashi in 1885 from *Ephedra sinica* Stapf. The latter, *ma-huang* 麻黃, Jap. *ma-ō*, has been used as a medicinal herb in China since well before the Christian era. Ephedrine is a powerful stimulant, and would thus be a more plausible preparation for warriors about to go into battle than the fly-agaric, which is a depressant. To list only the more salient effects on the mammalian organism, muscarine causes contraction of the pupils, depression of blood-pressure, cardiac slowing, increased peristalsis, and bronchial constriction: ephedrine causes dilation of the pupils, increase of blood-pressure, increase of heart rate, and relaxation of the muscles of the bronchi and gastrointestinal tract.³⁶ If the original Soma had been the fly-agaric, it would be extraordinary that the Iranians should have chosen as a substitute a plant which might have had entirely opposite effects.

To be fair, it must be added that not all the species of *Ephedra* contain the alkaloid; and of those which do, its concentration may vary considerably in relation to climatic conditions and geographical distribution. It would seem that much work remains to be done in botany, chemistry, and pharmacology before it will be sensible to make a further attack on the problem of the botanical identity of the Soma-plant.

Wasson writes (p. 69), 'India is a land where the incredible sometimes comes true, and I should be delighted, but not altogether surprised, to discover that there are still circles privy to the knowledge of the true Soma'. Few of us would now be surprised; and it may well happen that at some time in the future some Brahman will 'reveal' the secret that the Soma-plant was a red mushroom.

³⁴ Mary Boyce, 'Haoma, priest of the sacrifice', in M. Boyce and I. Gershevitch (ed.), *W. B. Henning memorial volume*, London, 1970, 62. The evidence quoted (*ibid.*, p. 64, n. 26) from Stein, *BSOS*, vi, 2, 1931, 502 ff., suggests, though it does not prove, that an *Ephedra* was already in use among the Central Asian Iranians in the fourth century A.D.—and not necessarily as Haoma.

³⁵ H. H. W. Pearson, *Gnetales*, Cambridge, 1929 (but written before 1916), lists nine or ten species whose geographical distribution might make them possible candidates; but his information is very imprecise. J. D. Hooker, *Flora of British India*, 1875–97 (information long out of date), v, 641, 863, found it extremely difficult to differentiate many of the species.

³⁶ Louis S. Goodman and Alfred Gilman, *The pharmacological basis of therapeutics*, second ed., New York, 1956.

But Wasson's book has now been published. If any such 'tradition' does come to be disclosed, we shall never know whether it has been fabricated from the book.

I cannot close without an expression of admiration for the enormous labour in scholarly research which Mr. Wasson has devoted to the preparation of this book,³⁷ and of gratitude for the great quantity of fascinating materials which he has placed before us. It is therefore with all the more regret that I find myself unable to accept that he has proved his theory that the original Vedic Soma was *Amanita muscaria*.

³⁷ See now also the review by F. B. J. Kuiper, *Indo-Iranian Journal*, xii, 4, 1970, 279-85, with further comments by R. G. Wasson, *ibid.*, 286-98, published after the present article had been sent for printing.

[Addendum. Mr. Wasson has very kindly sent me a copy of an article by P. Catalfomo and C. H. Eugster, '*Amanita muscaria*: present understanding of its chemistry', *Bulletin on Narcotics*, xxii, 4, 1970, 34-41. The authors show that 'the total muscarine content of *A. muscaria* is extremely low (0.0002 per cent on a fresh weight basis)'. Thus, some of my remarks on pp. 360-1, in so far as they concern muscarine, are probably not relevant for the fly-agaric problem. Unfortunately, this information reached me only after the present article had been set in pages. The paragraphs in question could not be rewritten without undue expense and delay in printing; and I am grateful to the Editorial Board for permitting me this additional note. It should be clear, however, that the chief point of my argument still stands, namely, the nausea, vomiting, and coma caused by the fly-agaric, even if the chemical agents responsible for these effects are not yet definitely decided by pharmacologists.]

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MICROBORE SINGLE-COLUMN ANALYSIS OF PHARMACOLOGICALLY ACTIVE ALKALOIDS FROM THE FLY AGARIC MUSHROOM *AMANITA MUSCARIA*

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SUMMARY

Microbore single column conditions are described which allow the separation and quantification of the substituted isoxazole alkaloids muscimol and ibotenic acid, the pharmacologically active principles of *Amanita muscaria*. The procedure permits the evaluation of levels of the alkaloids at all stages of purification from fungal extracts and provides a convenient quantitative method for monitoring the formation of muscimol by the decarboxylation of ibotenic acid.

INTRODUCTION

The remarkable halucinogenic and fly killing properties of the fly agaric mushroom *Amanita muscaria* have been known and exploited for centuries. The active constituents of the fungus were found to be ibotenic acid [amino(3-hydroxy-5-isoxazolyl)acetic acid] and its decarboxylation product muscimol (3hydroxy-5-aminomethylisoxazole)¹⁻⁵ (Fig. 1). These two compounds have, in recent years, come into prominence by virtue of their potent pharmacological effects in the mammalian central nervous system⁶. Ibotenic acid, a powerful glutamate agonist⁷, may be envisaged as a conformationally restricted glutamic acid. Muscimol has also neurotransmitter properties, being a powerful γ -aminobutyric acid agonist⁷, by virtue of its structural resemblance to γ -aminobutyric acid. Both compounds have recently received much attention as probes for investigating the nature of glutamate and γ -aminobutyric acid binding to their respective receptors and have been suggested as possible models for the development of potential drugs in the treatment of specific nervous disorders such as Huntington's Chorea and epilepsy.

Since the chemical syntheses of both ibotenic acid and muscimol are difficult⁸ it is often more convenient to isolate the compounds from the mushroom which is readily available.

A major problem arising during the purification of ibotenic acid and muscimol from the mushroom is one of determining the amounts of the alkaloids in crude extracts and of monitoring their levels during the isolation procedure. Large quan-

ities of other amino acids and ninhydrin positive compounds are present in the mushroom which co-chromatograph with the alkaloids on conventional thin-layer chromatographic (TLC) systems. In addition, the levels of the alkaloids vary with age and subspecies of the mushroom and it is important to have a knowledge of their level prior to the purification. Furthermore, the preparation of muscimol in our laboratory by the decarboxylation of ibotenic acid required an accurate, rapid and reliable means of determining the levels of two compounds.

In this paper we report a microbore single column method for the quantitative determination of both ibotenic acid and muscimol and its application during the purification of ibotenic acid from the mushroom. The method has been used to locate the two alkaloids in the mushroom and for monitoring the chemical decarboxylation of ibotenic acid into muscimol.

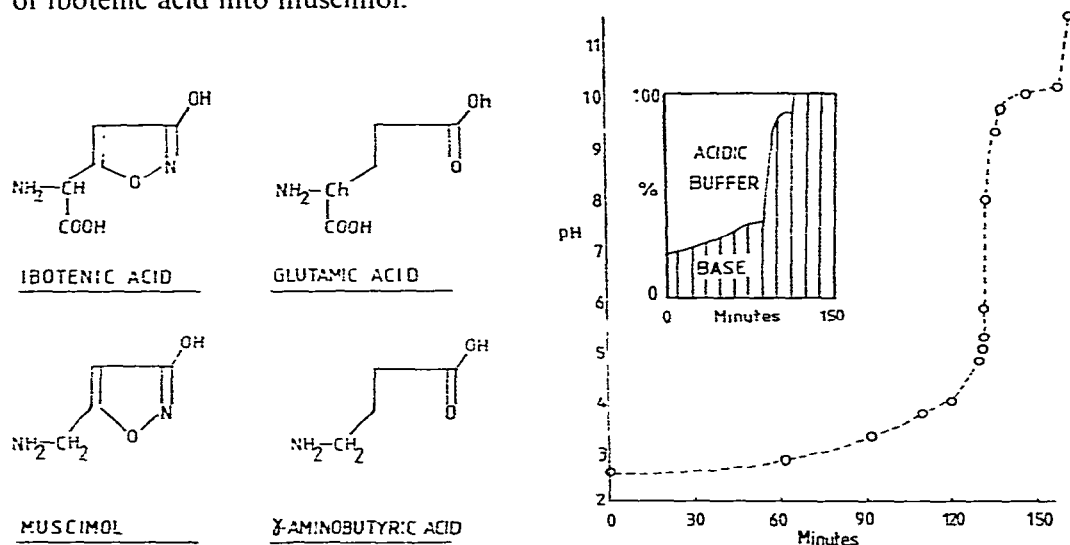


Fig. 1. The proposed conformationally restricted two-dimensional structures of glutamic acid and γ -aminobutyric acid and their structurally similar analogues, ibotenic acid and muscimol (respectively).

Fig. 2. A diagrammatic representation of the pH gradient employed obtained by mixing the acid and basic buffers (see Experimental) in the proportions indicated by the inset.

EXPERIMENTAL

Reagents

All reagents used are from BDH (Poole, Great Britain) and were of the Analar grade.

Sample preparation

Protein free extracts of *Amanita muscaria* were prepared by homogenising a given dry weight in 90% ethanol containing a known amount of the internal standard norleucine. After centrifugation at 30,000 g for 30 min the supernatant was taken, evaporated to dryness at 10°C and the dried extract redissolved in 20 mM HCl immediately before application to the column. Preliminary results suggested that the amounts of both ibotenic acid and muscimol measured were dependent on the acidity and temperature of this solution and the length of time between dissolving the extract

and applying the sample to the column. Further details of this phenomenon are given in the Results and discussion section.

Instrumentation

A Rank-Hilger J180 automated amino acid analyser was used to separate and detect the amino acids extracted from the *Amanita muscaria*. A single column system was employed (350 × 2.7 mm I.D.) and eluted amino acids were detected at 570 nm and 440 nm by reaction with ninhydrin in the presence of potassium cyanide as reducing agent. The buffer used to separate the various amino acids was generated "in situ" by mixing an acidic and a basic solution together in various ratios to give a pH gradient described in Fig. 2. The composition of the two buffers used for the elution process and for the salt free buffer used while loading the samples are given below.

Buffers

"Salt-free" loading buffer (pH 2.1). 21.0 g citric acid, 100 ml methanol, 2.5 ml thioldiglycol (25%, w/w, in water), 3.5 ml of a 10% Brij 35 solution and distilled water to 1 l.

Acidic buffer (pH 1.9). 21.0 g citric acid, 11.7 g NaCl, 50 ml isopropanol, 2.5 ml thioldiglycol (25%, w/w, in water), 3.5 ml of a 10% Brij 35 solution all made up to 1 l with distilled water.

Basic buffer (pH 11.9). 19.0 g disodium tetraborate, 4.2 g NaOH, 10 ml of a 4% EDTA solution, 3.5 ml of a 10% solution of Brij 35 all made up to 1 l with distilled water.

Ninhydrin. 10 g ninhydrin, 216 g sodium acetate trihydrate, 400 ml α -methoxyethanol, 100 ml glacial acetic acid, 10 ml of 10% solution of Brij 35 were dissolved in distilled water and the volume made up to 1 l.

Reducing agent. 1 ml of a 1% solution of KCN was diluted into a stock solution of 1 l of distilled water containing 5 ml of 4 M NaOH and 3.5 ml of 10% Brij 35.

Colour development. The eluent from the chromatographic column (0.2 ml/min) is mixed with the ninhydrin solution (0.2 ml/min) and cyanide solution (0.025 ml/min). A nitrogen bubble is used to segment the stream (1 bubble every 2 sec) and the resultant mixture incubated at 90°C for 7 min prior to passing through the colorimeter where the absorbance at 570 nm and 440 nm was measured.

Quantitation of amino acids

Purified ibotenic acid (monohydrate, mol.wt. 176) and muscimol (free base, mol.wt. 125) were weighed out and their colour coefficients in the ninhydrin complex estimated relative to that of the norleucine-ninhydrin complex. These calculated "colour coefficients" were then used to quantitate these amino acids in elution profiles of extracts from *Amanita muscaria* as an estimate in purification or biosynthetic experiments.

RESULTS AND DISCUSSION

A reliable and quantitative separation of ibotenic acid and muscimol could be achieved using a microbore column and a citrate buffer elution system as described in the legends to Figs. 3 and 4.

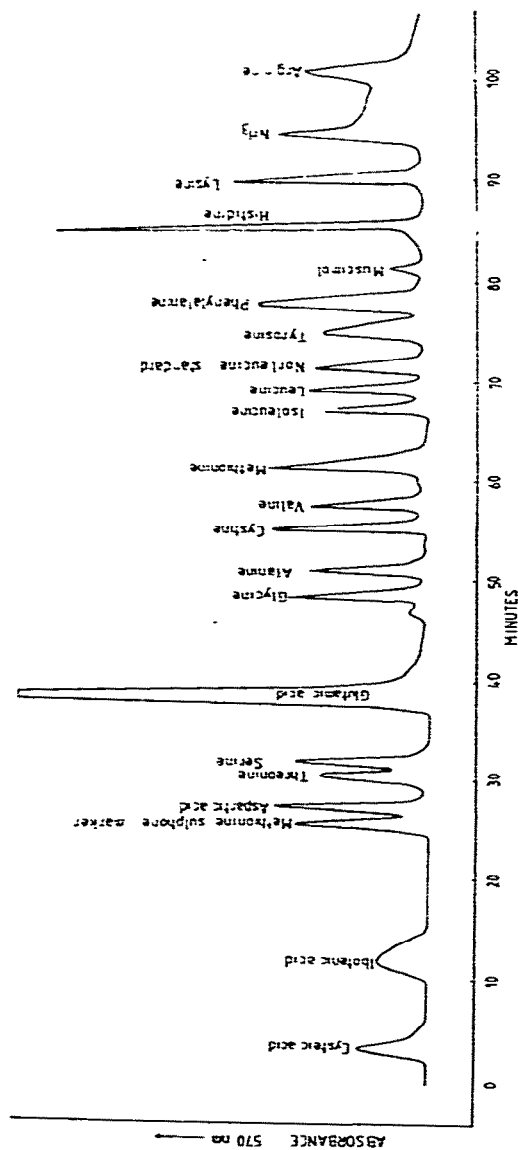


Fig. 3. An elution profile of a standard mixture of amino acids also containing ibotenic acid and muscimol. All amino acids were present at 10 nmol/ml in the sample with the exception of L-glutamate which was present at 50 nmol/ml.

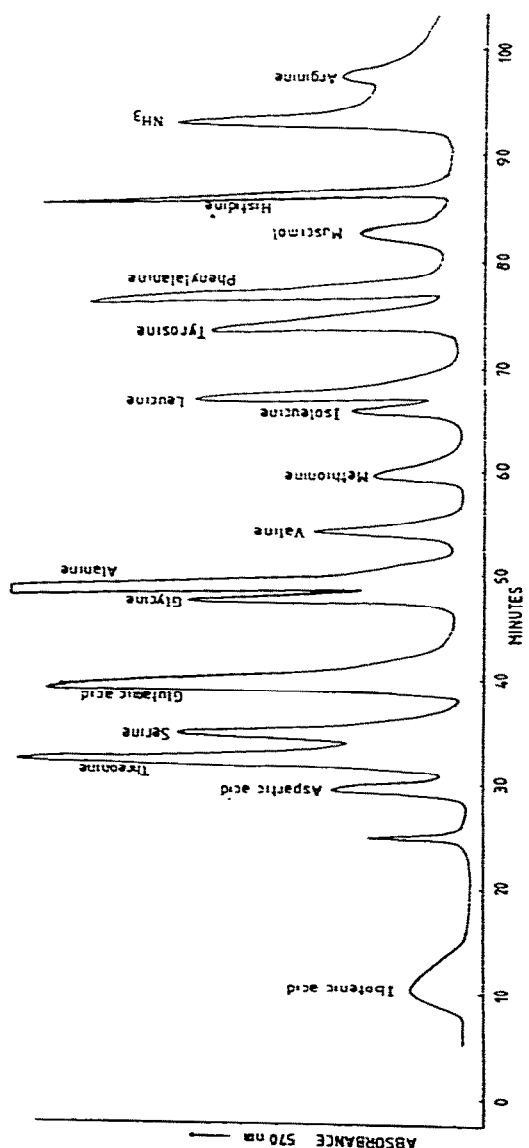


Fig. 4. An elution profile given by a protein free extract of *Amanita muscaria*. Dried fungus was deproteinised by 95% ethanol and the supernatant from a 30-min centrifugation at 20,000 g was evaporated to dryness and the amino acids redissolved in 20 mM HCl (500 μ l).

The loading buffer system employed was at an initial pH of 1.8 to enhance the binding of the ibotenic acid. Under the conditions used, ibotenic acid had a retention time of 11 min and muscimol chromatographed near histidine with a retention time of 83 min, Fig. 3. The colour coefficients for both compounds determined under the above conditions were 0.42 and 0.48 for ibotenic acid and muscimol respectively relative to the standard norleucine (colour coefficient = 1.0). Although the ibotenic acid is retained by the resin, the affinity is relatively weak as exemplified by early elution from the column and the tailing of the peak, Fig. 4. Attempts to lower the pH to increase binding of ibotenic acid promotes the partial decarboxylation to muscimol. The pH chosen thus represents a compromise which allows satisfactory binding without decomposition. The method was used to determine the levels of ibotenic acid and muscimol in different parts of the mushroom, *Amanita muscaria* (Table I). The yellow region under the red cap cuticle of the mushroom has the highest concentration of ibotenic acid (548 nmol/g) with the white flesh below also containing substantial quantities (153 nmol/g). Knowledge of the distribution of the alkaloids was of great importance in the purification of the compounds since it allowed the portions of the mushroom richest in the alkaloids to be removed prior to homogenisation, thus greatly lowering the amount of starting material required.

The method was also of use for determining the levels of ibotenic acid and muscimol in the whole mushroom and for monitoring the purity of the isolated alkaloids with respect to amino acid impurities (Figs. 3 and 4).

Ibotenic acid is unstable in crude extracts or in acid due to enzymic and non-enzymic decarboxylation to muscimol. Fig. 5 shows the result of the decarboxylation process proceeding under mild conditions. Here 50 nmol of ibotenic acid were incubated for 0 min (Fig. 5a) or for 240 min (Fig. 5b) in 20 mM HCl at room temperature (21°C). The acid catalysed decarboxylation of ibotenic acid to muscimol is therefore a source of error in the estimation of either amino acid if for example an acidic protein precipitation procedure precedes the estimation. Under the conditions of Fig. 5 the yield of muscimol is almost quantitative with no indication of unwanted side products.

TABLE I

DISTRIBUTION OF IBOTENIC ACID AND MUSCIMOL IN MATURE MUSHROOM OF *AMANITA MUSCARIA*

Weighed amounts of tissue from various parts of the mushroom were homogenised in two volumes of water. The homogenate was stirred for 30 min and then centrifuged for 30 min at 20,000 g. An aliquot (100 μ l) of the supernatant was deproteinized by the addition of 400 μ l of ethanol and after removal of the precipitate the supernatant was freeze dried. The residue was redissolved in 20 mM HCl (500 μ l) prior to microbore column analysis. Values quoted are nmol/g wet weight tissue and are calculated from the area of the trace as described.

<i>Tissue sample</i>	<i>Ibotenic acid</i> (nmol/g wet weight)	<i>Muscimol</i> (nmol/g wet weight)
Pigmented cuticle	None detectable	113.9
Yellow flesh	548.0	366.0
White flesh	153.0	308.7
Gill	73.0	365.0
Stalk —cross section	48.4	79.9

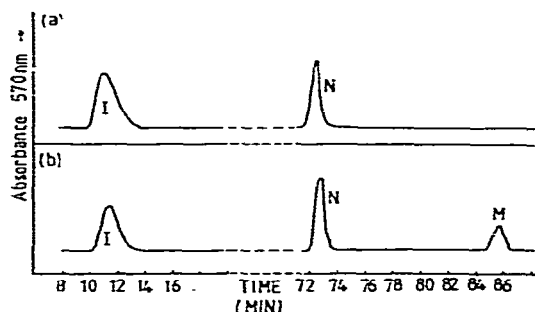


Fig. 5. Evidence of acid catalysed decarboxylation of ibotenic acid to muscimol. A solution of ibotenic acid 50 nmol/ml and norleucine (20 nmol/ml) in 20 mM HCl (21°C) was analysed immediately (a) and then after 240 min at 21°C (b). Trace b shows a peak co-chromatographing with muscimol. Peaks: I = ibotenic acid; N = norleucine; M = muscimol.

In summary we have developed a reproducible and quantitative method for the determination of the alkaloids ibotenic acid and muscimol which is sensitive enough to detect down to 30 ng of compound, and which allows the estimation of the alkaloids in crude extracts of *Amanita muscaria*. The method has the potential of further development for use in the estimation of the alkaloids in biological fluids such as blood or urine thus allowing the evaluation of kidney clearance or metabolism of the compounds under *in vivo* experimental conditions.

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13. 2,3- and 4,5-Secodopa, the Biosynthetic Intermediates Generated from L-Dopa by an Enzyme System Extracted from the Fly Agaric, *Amanita muscaria* L., and Their Spontaneous Conversion to Muscaflavin and Betalamic Acid, Respectively, and Betalains

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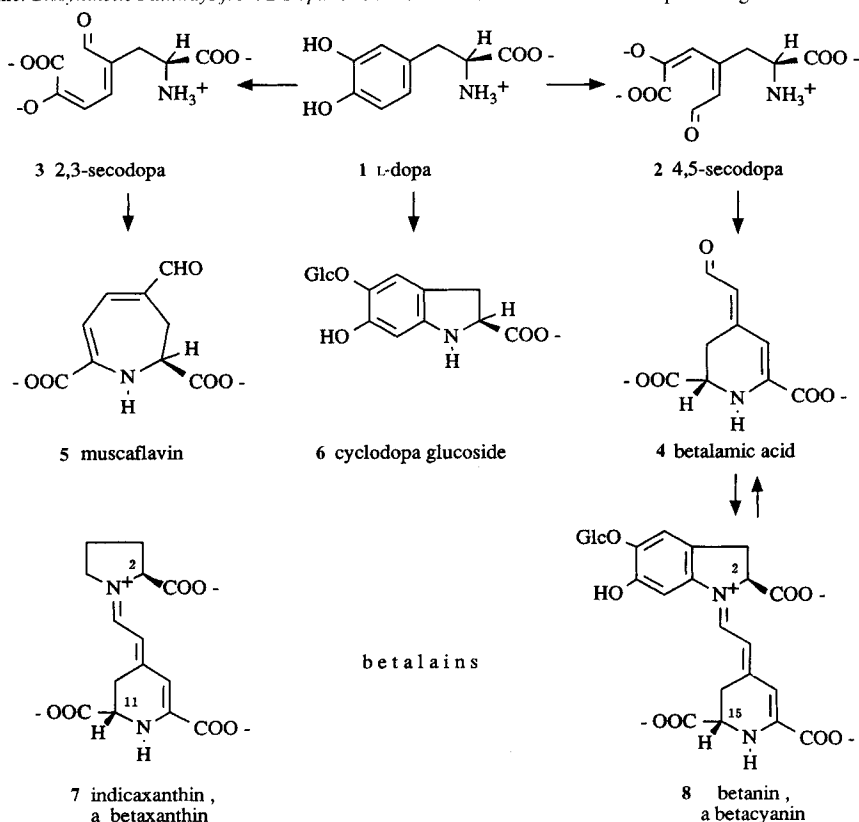
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An enzyme is extracted from the red peel of *Amanita muscaria* which cleaves the C(2)–C(3) and the C(4)–C(5) bond of the aromatic ring of L-dopa (**1**) to form a mixture of 4,5-secodopa (= salt of 6-amino-2-hydroxy-4-(2'-oxoethylidene)hept-2-enedioic acid; **2**) and 2,3-secodopa (= salt of 7-amino-5-formyl-2-hydroxyocta-2,4-dienedioic acid; **3**), two hitherto hypothetical biosynthetic intermediates (see *Scheme*). Though isolation of these products has not been possible, structural evidence is inferred from reaction products, kinetics, and spectroscopical characteristics in comparison with known compounds. Secodopas **2** and **3** are characterized in dilute solution by HPLC and UV/VIS spectroscopy (anions: $\lambda_{\max}$ 424 and 414 nm, resp., ϵ_{420} = 25 500; on acidification, shift to 380 and 372 nm, resp.). They cyclize without enzyme catalysis, optimally at pH 4.5–5; **3** produces muscaflavin (**5**) and **2** betalamic acid (**4**). The products are identified by direct comparison with authentic samples in HPLC, by ¹H-NMR of **5**, and by condensation of **4** with L-proline to form the well known betalain indicaxanthin (**7**). The enzymatic conversion of L-dopa (**1**) *via* **2** to betalamic acid (**4**; (*S*)) and its condensation with L-proline leads to pure natural indicaxanthin (**7**; (2*S*,11*S*)); correspondingly, the enzymatic conversion of D-dopa to (*R*)-betalamic acid and its condensation with L-proline produces isoindicaxanthin ((2*S*,11*R*)) which is unknown in nature. Particularly relevant is the fact that the same enzyme cleaves pyrocatechol to produce a solution of the enolate form of the known 2-hydroxy-6-oxohexa-2,4-dienoate (secopyrocatechol; **9**; see *Fig. 5*). Dissociation constants of the corresponding enolic functions in the cleavage products are determined by spectrometric titration and compared to those of known systems.

Introduction. – In the early sixties, it was realized that the vacuolar coloring system of plants is not entirely supported by anthocyanins as presumed previously. A group of families of the order *Centrospermae* uses a special palette of colorations covered by the red-violet betacyanins and the yellow betaxanthins [1a]. The originality of this choice lies in the fact that betacyanins and betaxanthins, coined betalains as a class [1b–f], are built by the same principle, the condensation of a merocyanin, betalamic acid (**4**), with amino acids to form cyanin dyes (*Scheme*). Typical representatives are the red-violet betanin (**8**) [2] of beetroot containing the unusual indoline amino acid cyclodopa glucoside (**6**) [3], and the yellow indicaxanthin (**7**) of cactus flowers or fruits containing proline [4]. Betalamic acid (**4**) has been found also free [5a] in association with betaxanthins [5b]. Surprising in this context is the fact that betaxanthins, considered taxonomically characteristic for the mentioned plant families, are produced as well in the isolated case of some fungi of the *Agaricales*, in particular in the toadstool fly agaric, *Amanita muscaria* [6].

¹⁾ Part of the Ph. D. thesis of F.T., Université de Lausanne, 1989.

Scheme. Biosynthetic Pathways from L-Dopa. The metabolites are shown in the form prevailing in neutral medium.



A specially attractive chapter has been the biosynthetic aspect of the betalains. It was suggested [7] that betalamic acid (4) arises from L-dopa (= 3-hydroxy-L-tyrosine; 1) by cleavage of the C(4)–C(5) bond of the aromatic ring to form an intermediate which by cyclization leads to 4 (Scheme), and that the formation of cyclodopa started as well from 1. The proposed pathway received experimental support from incorporation studies in red beet and cactus fruits [8].

The presumed cleavage product 2 of dopa which we propose to name 4,5-secodopa²⁾ has not yet been noticed in the plants. A precedent of this type of enzymatic ring cleavage is the action of enzymes from strains of *Pseudomonas* on simple catechols as shown by Dagley and Hayaishi [9] [10]; the cleavage was found to take place at the bond adjacent to the diol group, called the 'extradiol' position, and the enzymes have been classified as metapyrocatechases. Corresponding enzymes in betalain-producing plants have not yet been seized.

It appeared promising to extend the biosynthetic investigation on the formation of betalains to the fly agaric *Amanita muscaria*, especially in view of the occurrence there of

²⁾ The prefix 'seco' has been applied in steroid nomenclature in a different sense, as defined by IUPAC rule S-7.4. The use of 'secodopa' as a trivial name has some precedent in the field of alkaloids, however.

an isomer of betalamic acid (**4**), muscaflavin [6a] (**5**); this compound is assumed to be the cyclisation product of 2,3-secodopa (**3**), another possible 'extradiol'-cleavage product of L-dopa (**1**). Muscaflavin may serve as additional argument in support of the biosynthetic hypothesis and has been looked for in *Centrospermae* plants without success [5b]. We noticed that red or yellow tissue parts from the cap of the fly agaric incubated with **1** at pH 7 developed yellow solutions absorbing near 420 nm; these solutions contained effectively, among other products, **4** and **5** offering the unique occasion to undertake this investigation which led us to the discovery of the intermediate secodopas.

Results. – Enzyme Preparation and Activity. The red epidermis peeled off the cap of *Amanita muscaria* is ground under liquid N₂, and the enzymes are extracted from the powder at 4° and pH 8. Some inactive protein part is removed by precipitation with 30% acetone/H₂O; the active enzyme is precipitated in a second fraction increasing the acetone part to 60%. Further purification steps have not been achieved; because of its instability, the enzyme preparation has been used freshly. The enzymatic activity in incubation tests with L-dopa (**1**) at pH 8 is measured from the initial linear increase of absorbance at 420 nm of the developing yellow solution. The enzyme remains well conserved in the frozen untreated tissue at –30°. But an extracted preparation left at 25° loses within 1 h ca. 20% of its activity. Loss of activity may be limited at 4° to 1% per h, and preparative assays are, therefore, performed under this condition.

The enzyme is remarkably active towards some other catecholic substrates. Comparing the initial rates of enzymatic action to that on L-dopa taken as 1.0, the relative rates of other substrates are for D-dopa 0.62, α -methyldopa 1.12, dopamine 1.69, *N*-acetyldopamine 0.13, pyrocatechol 0.19, and 3,4-(dihydroxyphenyl)acetate 0.04; the enzyme is inactive among others towards 3-(3,4-dihydroxyphenyl)propionate.

Products of Enzymatic Action on L-Dopa. Four products **2–5** are formed in the yellow solution of the enzymatic assay with **1** which are separable by HPLC on reversed-phase ODS Hypersyl with a gradient of phosphate buffer pH 6 and MeOH (detection at 420

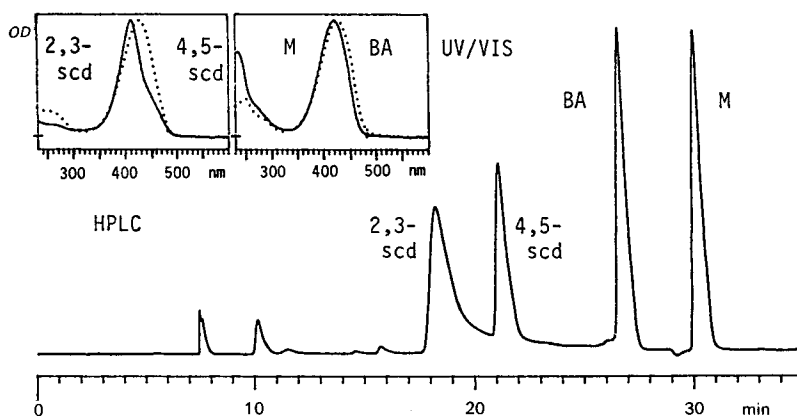


Fig. 1. HPLC and UV/VIS spectra of the products **2–5** formed during incubation of L-dopa (**1**) with the enzyme extract from the peel of *Amanita muscaria*. HPLC: ODS Hypersyl (5 μ); 0.05M Na₂HPO₄ buffer pH 6 containing 0.03M (Bu₄N)H₂PO₄ with a 0–40% MeOH gradient within 25 min, then isocratic for 10 min; detection at 420 nm. UV/VIS spectra are taken at the product peaks; 2,3-secodopa (**3**; 2,3-scd, —) and 4,5-secodopa (**2**; 4,5-scd, ·····), muscaflavin (**5**; M, —), and betalamic acid (**4**; BA, ·····).

nm; Fig. 1). The products ratio varies with time and pH as will be outlined later. The peaks at t_R 27 and 30.5 min are attributed to betalamic acid (**4**) and muscaflavin (**5**), respectively, in a direct comparison with authentic samples (retention times, co-HPLC). These are provided by known methods: **5** by chromatography of a pigment extract from *Amanita muscaria* following Döpp and Musso [12], and **4** by hydrolysis of betanin (**8**) with ammonia [5a] [11] and chromatography. The enzymatic products **4** and **5** have UV absorptions at 430 and 422 nm (Fig. 1) corresponding to those of the authentic samples.

In repeating the procedure of Döpp and Musso [12] to obtain **5** from the pigment extract (Fig. 2), we arrive at an essential addendum. The separation of the pigment mixture by ion-exchange chromatography on DEAE-Sephadex A-25 with a NaCl gradient leads well to the pattern of peaks described, but owing to HPLC, it becomes evident that the zone assigned to **5** is actually composed of two overlapping components; these are separable, at least partially, by fractionation; the front fractions contain prevalently **4** and only the tail fractions pure **5**.

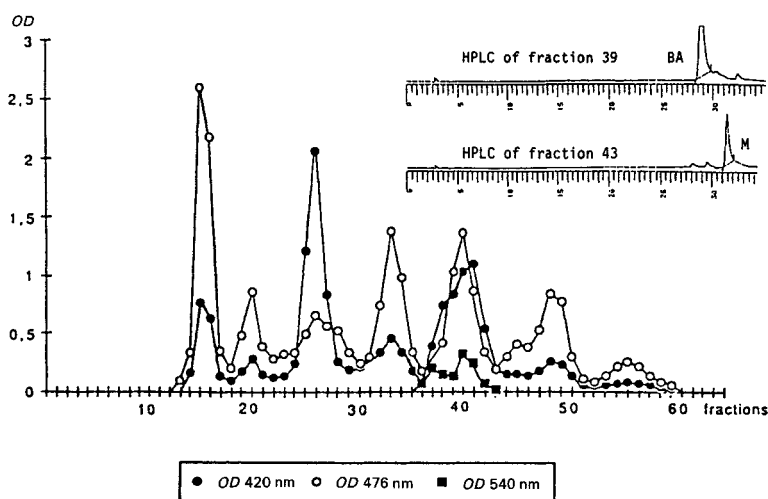


Fig. 2. Isolation of betalamic acid (**4**) and muscaflavin (**5**) from a methanolic extract of the red peel of *Amanita muscaria*. Elution profiles of the chromatography on DEAE-Sephadex A-25 (analogue to [12]) established from spectrophotometric readings at 420, 480, and 540 nm, and identification of the components in the front and tail fractions of the muscaflavin-containing zone by HPLC (conditions: see Fig. 1).

The relationship of the products **2** and **3** of the enzymatic assays to the identified ones (**4** and **5**) is established in an experiment. For this purpose, the enzymatically generated mixture of the cleavage products (**2** and **3**) is separated by HPLC; when the fractions containing the pure components are left for several h at pH 5.5, **3** is converted to muscaflavin (**5**) and **2** to betalamic acid (**4**), as established by HPLC. Hence there is a strong indication that **2** and **3** are the hypothetical intermediates 4,5- and 2,3-secodopa, and further kinetical, chemical, and spectroscopical arguments will consolidate this conclusion.

Recognition of the Secodopas as Intermediary Products from Kinetics. The evolution of products in enzymatic assays with L-dopa (**1**), incubated at 4° at different pH's, is followed by HPLC analysis of daily samples evaluating spectroscopically the separated

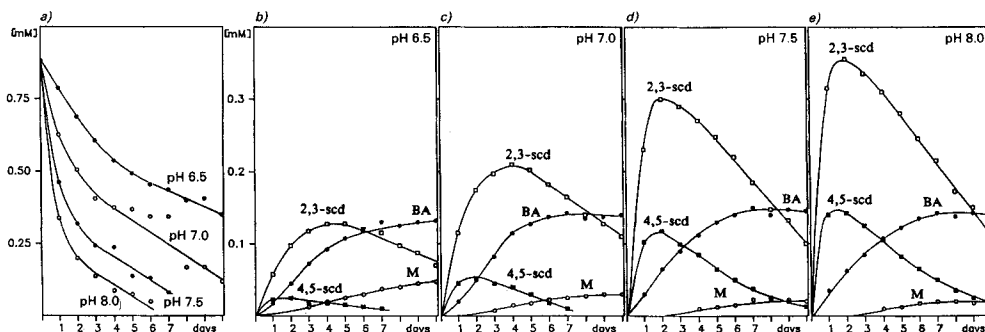


Fig. 3. Product developments after incubation of L-dopa (**1**) with the enzyme extract from *Amanita muscaria* at different pH's. The molar concentrations in the graphs are based on integrated absorbance peak areas of HPLC (Table 4 in *Exper. Part*) and calculated using known molar absorptivities for **1** ($\epsilon_{280} = 2800$), **4** and **5** ($\epsilon_{420} = 30\,000$), and an extrapolated value for the unknown **2** and **3** ($\epsilon_{420} = 25\,500$, see *Exper. Part*). a) Consumption of L-dopa (**1**) at different pH's; b–e) formation of products **2**–**5** at different pH's; 2,3-secodopa (**3**; 2,3-scd, $\square$ – $\square$ – $\square$), 4,5-secodopa (**2**; 4,5-scd, $\blacksquare$ – $\blacksquare$ – $\blacksquare$), muscaflavin (**5**; M, $\circ$ – $\circ$ – $\circ$), and betalamic acid (**4**; BA, $\circ$ – $\circ$ – $\circ$).

peaks of **1** and the four new compounds **2**–**5**. Fig. 3 shows the general picture of two parallel series of consecutive transformations $\text{dopa} \rightarrow \text{secodopas } \mathbf{2} \text{ and } \mathbf{3} \rightarrow \text{products } \mathbf{4} \text{ and } \mathbf{5}$, as will be explained in the following. The decrease of **1** appears to be exponential during the first days and then linear, and the fraction of enzymatically converted **1** is increased at higher pH (Fig. 3a). Graphically determined initial decay rates of **1** (Table 1) increase linearly with pH in the range 6.5 to 8 (decay should be zero near pH 6).

Table 1. Initial Rates of Enzymatic Dopa Decay and Secodopa Formation. Graphically evaluated from Fig. 3 in mM/d.

pH	L-Dopa (1)	4,5-Secodopa (2)	2,3-Secodopa (3)	Total secodopa
6.5	– 0.1	0.04 (40%)	0.06 (60%)	0.1
7.0	– 0.27	0.07 (35%)	0.13 (65%)	0.20
7.5	– 0.47	0.17 (37%)	0.28 (63%)	0.45
8.0	– 0.74	0.24 (32.5%)	0.50 (67.5%)	0.74

The curves of formation of the two primary products **2** and **3** (Fig. 3b–e), suspected to be 4,5- and 2,3-secodopa, are of complex character, initial rapid increase similar to that of a first-order reaction, turning into a decrease which remains linear over several days. The total extent of this development is enhanced with pH (Table 1). The graphically evaluated initial rates of formation of the secodopas sum up approximately to that of L-dopa consumption confirming that the two events must be concomitant. The ratio of these rates of formation indicates fractions of *ca.* 35% of 4,5-secodopa (**2**) and *ca.* 65% of 2,3-secodopa (**3**). The rates of linear decay are proportional to the maximum amounts of the intermediates formed. The formation of the final products, **4** and **5**, delayed by an initial lag time, progresses at clearly different rates. Muscaflavin (**5**) remains very much below expectation and is even decreased with raising pH in contrast to the observed complementarity of the preceding transformation $\mathbf{1} \rightarrow \mathbf{3}$; an important part of **3** has disappeared to unknown products. The development of betalamic acid (**4**) is rather

important in proportion and nearly identical over the examined pH range, irrespective of the amount of preceeding **2**.

A supplementary information is obtained by following the transformation of the intermediates to the final products independently. Starting material is a mixture of secodopas **2** and **3**, which may be generated efficiently by a short-time enzymatic assay at pH 8. Such samples are incubated at pH's between 3 and 6.5. Within the initial 12 h, the decay of **2** and **3** exhibits each the characteristics of a first-order reaction. Similar samples which are deproteinated by addition of acetone after the enzymatic generation of **2** and **3** develop in the same way as in presence of the proteins, confirming that this conversion does not need enzyme catalysis. The formation of the final products **4** and **5**, respectively, exhibits as well characteristics of a first-order reaction but is by far not complementary to the decay of the corresponding secodopa. An illustrative view of the development within the first 2 h is given in a bar chart (Fig. 4) where the amounts of the disappeared

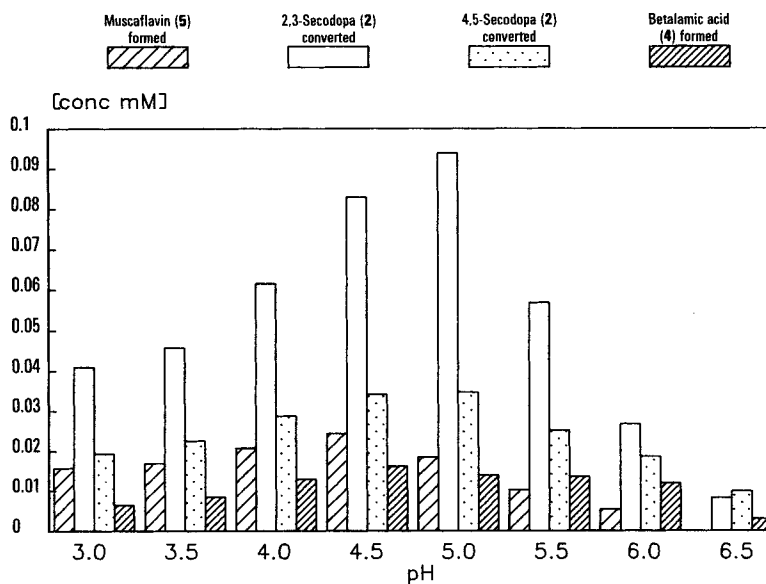


Fig. 4. Conversion of 2,3- and 4,5-secodopa (**3** and **2**, resp.) to muscaflavin (**5**) and betalamic acid (**4**). The concentrations of **2–5** are established by HPLC 2 h after incubation at the different pH's.

secodopas are compared to those of products formed. The overall profile of the disappeared amounts of **2** and **3** in function of pH is a bell-shape curve with a maximum between 4.5 and 5. The amounts of final products formed remain below those of their disappeared precursors. The optimum of product formation appears all the same to take place in the same pH range near 4.5. Remarkable is the proportion product/disappeared precursor which is generally higher for **4** than for **5**; apart from that, formation of **5** is relatively favored under acidic condition and negligible above pH 6, and that of **4** is relatively enhanced towards the neutral range.

Formation of Betaxanthins and Enantiomeric Purity of Enzymatically Generated Betalamic Acid (4). The addition of L-proline to the enzymatic assay leads to a characteristic

change of the kinetic picture. Only a limited amount of betalamic acid (**4**) shows up and vanishes, and in its place, a new final product absorbing at 480 nm is formed in important yield; no change is observed in the development of muscaflavin (**5**). The identity of the new product as indicaxanthin (**7**) is established by HPLC at pH 2.8³⁾ in a direct comparison with an authentic sample synthesized from betanin (**8**) [11]. In fact, the synthetic sample has been prepared from a 67:33 mixture of **8** (15*S*) and its (15*R*)-isomer isobetanin, thus giving rise to two very close peaks in the HPLC, the smaller one appearing first (35%; isoindicaxanthin) followed by the bigger one (65%; **7**); the peak of the enzymatically generated product matches perfectly to the latter one.

The stereochemical implication of this result is confirmed in a complementary experiment. If an enzymatic assay with L-dopa (**1**) in presence of L-proline produces pure indicaxanthin ((2*S*,11*S*); **7**), the corresponding assay with D-dopa should lead to isoindicaxanthin ((2*S*,11*R*)). This is indeed the case, since an enzymatic assay with D-dopa in presence of L-proline produces, according to HPLC, a single compound (besides **5**) which is identical to the synthetic isoindicaxanthin (see above). Confirmation is given by an enzymatic assay with DL-dopa and L-proline which produces a 42:58 mixture (HPLC) of isoindicaxanthin and **7** (the unequal distribution might reflect the different reactivity of the enzyme towards the two enantiomers). The mixture obtained from DL-dopa and L-proline has served as HPLC standard; when it is mixed with an equal amount of mixture from the enzymatic assay with D-dopa and L-proline, the area of the isoindicaxanthin peak is enhanced, as expected from a perfect addition of the corresponding product areas. Similarly, the peak of **7** is enhanced upon addition of the product solution resulting from the assay with L-dopa (**1**) and L-proline. This experiment provides a double proof: it confirms, on one hand, the identity of betalamic acid (**4**) and, on the other hand, establishes the optical purity of enzymatically generated **4** from optically pure substrate.

An artifact is produced when *Tris* buffer is used in the enzymatic assays instead of phosphate buffer; a new product shows up (HPLC: t_R 10 min), which exhibits a UV/VIS spectrum characteristic of a betaxanthin (λ_{max} 476 nm); isolated, it liberates betalamic acid (**4**) on alkaline hydrolysis. It may be concluded that the new product is *Tris*-betalain⁴⁾ a cyanine dye resulting from the condensation of the buffer amine with **4**. In presence of L-proline at even low concentrations, the production of *Tris*-betalain is largely suppressed, and that of indicaxanthin (**7**) becomes prevalent⁵⁾.

Isolation of Enzymatically Generated Muscaflavin (5). A combined procedure is used to prepare **5** in sufficient amount for ¹H-NMR characterization: A mixture **2/3**, enzymatically generated at pH 8, is acidified to pH 5.5 which accelerates cyclization. By addition of L-proline, the less stable **4** is converted entirely to **7**, a salt which is not extracted by organic solvents. Thus, **5** can now be extracted at acidic pH and is further purified on *Sephadex LH-20*. The ¹H-NMR spectrum (CD₃OD) of the brownish-yellow product

³⁾ Gradient and pH for efficient separation of betaxanthins are different from those in *Figs. 1* and *2*; see *Exper. Part* for details.

⁴⁾ For nomenclature, see [8].

⁵⁾ The presence of still another product is observed with increasing concentrations of L-proline, at the expense of muscaflavin (**5**) formation. To this product (λ_{max} 438 nm), we provisionally assign the structure of a cyanine dye or merocyanin, condensation product of 2,3-secodopa (**3**) with L-proline.

corresponds to the one reported by *Musso et al.* [13] for synthetic **5** (salt in H₂O), except for some difference in the chemical shifts.

Spectrometric Titrations. The yellow, relatively stable neutral solutions of **2/3** display intense absorption maxima in the visible, 4,5-secodopa (**2**) at 424 nm and 2,3-secodopa (**3**) at 414 nm. On acidification, the maxima shift by 42–44 nm to 380 and 372 nm, respectively; the spectra of these protonated forms, however, fade away rather rapidly, and are available only by quick recording techniques. UV/VIS spectra recorded within the pH range of titration (*Fig. 5a, b*) provide data composed of those of the protonated

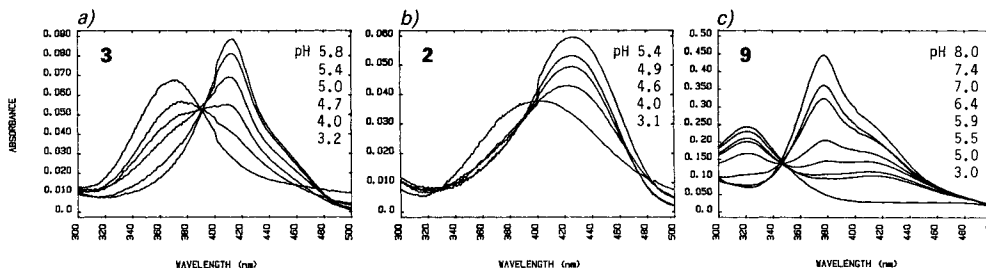


Fig. 5. UV/VIS spectra of enol and enolate forms of a) 2,3-secodopa (**3**), b) 4,5-secodopa (**2**), and c) secopyrocatechol (**9**) in the pH range of titration. The enol-enolate equilibrium represents the acid-base pair concerned in the titration. Deprotonation involves a red shift of the absorbance maximum.

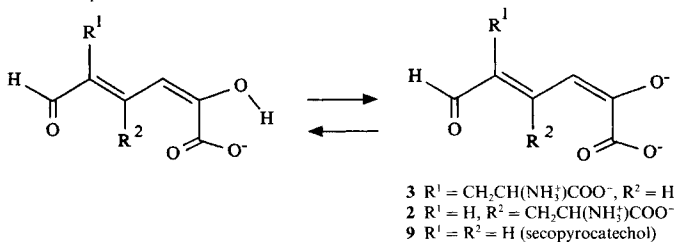


Table 2. UV Absorptions and pK_a-Values of Glutaconaldehyde Derivatives

	$\lambda_{\max}$ [nm] of AH form	$\lambda_{\max}$ [nm] of A ⁻ form	pK _a
2,3-Secodopa (3)	372 (pH 3.2)	414 (pH 5.8)	5.0 ± 0.1
4,5-Secodopa (2)	380 (pH 3.1)	424 (pH 5.4)	4.8 ± 0.1
Glutaconaldehyde (pent-2-enedial)	305 [14]	362	5.75 [16]
Secopyrocatechol (9 ; see <i>Fig. 5</i>)	322 (pH 3)	378 (pH 8)	6.7 ± 0.1

and anionic forms with a characteristic isobestic point; pK_a values are evaluated from these series by means of a graphical linearization method⁶⁾ (*Table 2*).

Most relevant is a comparison of these spectra with that of secopyrocatechol²⁾ (= 2-hydroxy-6-oxohexa-2,4-dienoate; see *Fig. 5, 9*), the known [9] cleavage product from pyrocatechol (= benzene-1,2-diol). A solution of **9** is easily generated by incubation of the enzyme extract from *Amanita muscaria* with pyrocatechol. It can be shown that the

⁶⁾ Since uncertainty persists concerning the 'acidic end' of the spectra series, a linearization method is chosen that refers to the 'alkaline end' of titration. The pK_a data obtained are still to be considered approximately.

cleavage of pyrocatechol is accomplished by action of the same enzyme as that for L-dopa (**1**) since in assays with mixtures of **1** and pyrocatechol (*Table 7*, see *Exper. Part*), a depression of secodopa formation is noticed⁷⁾. The shape of the UV/VIS spectrum of **9** at various pH is strikingly similar to that of 2,3-secodopa (**3**), except for the position of the maxima, giving testimony of close structural relationship (see *Fig. 5c*). On passing from the enol to the enolate form, the absorption maximum shifts quite similarly to the red by 55 nm; such a shift is also reported for glutaconaldehyde [14]. The pK_a of the enolic system **9**, 6.7, determined similarly from a spectra series in the titration range (*Fig. 5c* and *Table 2*), indicates weaker acidity than that of the secodopas.

Discussion. – The present work contributes to clarify the essential steps in the biosynthetic conversion of L-dopa (**1**) to the betalains (*Scheme*); *i.e.* the enzymatic formation of the secodopas **2** and **3** from **1**, their spontaneous cyclization to the known products muscaflavin (**5**) and betalamic acid (**4**), and the formation of betalains from **4** under neutral conditions.

The temporary existence of the secodopas **2** and **3** is demonstrated for the first time. We gathered substantial evidence for their identity despite the only limited amounts available in the dilute solutions of the enzymatic assay. Usual methods for the determination of structure are not feasible due to the instability of **2** and **3**. The strongest argument for their structure is the comparison with the known cleavage product **9** from pyrocatechol, produced by the same enzyme from *Amanita*; but in contrast to **9** which is stable in weakly alkaline medium and can be isolated, the tendency of **2** and **3** to cyclize makes their isolation problematic. On the other hand, the disappearance of **2** and **3** in acid medium yielding unidentified products is not unexpected in view of the known reactivity of similar glutaconaldehyde systems [15]. Plausible reasons why these primary intermediates have not been noticed before may be either their presence in minute amounts in the plant or their loss during the extraction procedure. The question of their occurrence in nature can now be examined profiting of HPLC characterization.

Noteworthy is the fact that the enzyme from *Amanita muscaria* acts as a metapyrocatechase on various substrates, whereas the activity of known metapyrocatechases of bacterial origin remains restricted to simple catechols. Considering the assortment of tested substances, the presence of an amino group appears to be essential for a good substrate. The enzyme of *Amanita* is obviously less stable than those of bacterial or animal origin [10b]. Whether the cleavage by the enzymes from *Amanita* at both 'extra-diol' sites of L-dopa (**1**) is due to the action of one non-regiospecific enzyme or a mixture of two isoenzymes is an open question; the fact that in *Centrospermae* plants, exclusively 4,5-bond cleavage of **1** takes place advocates rather in favor of the existence of isoenzymes.

Information on the acidity of hydroxydienones such as those contained in the cleavage products of **1** and pyrocatechol has been lacking. This is certainly due to technical problems in spectra recording (now obsolete) caused by the instability of the protonated forms of such dienol systems. The only dissociation constant of this kind is that of glutaconaldehyde (pK_a 5.75) which was determined with admirable experimental effort [16].

⁷⁾ We wish to acknowledge a helpful discussion with Bruce Ganem.

Illuminating is a comparison of the acidities of the secodopas **2** and **3** to those of known similar chromophors. Secopyrocatechol (**9**; pK_a 6.7) is a weaker acid than the unsubstituted glutaconaldehyde which can be readily interpreted as a consequence of chelation by the carboxylate group. Chelation should also be operative in the case of **2** and **3**, but in contrast, enhanced acidities (pK_a 4.8 and 5, resp.) are noted. The enolate form of **2** and **3** is thus stabilized by an effect which outweighs the contrarious effect due to chelation of the enol. This could be either a consequence of substitution of the chromophoric system or due to mutual interaction with the ammonium group in the alanine side chain (H-bonding) of **2** and **3**, an effect similar to that encountered in amino acids (see *e.g.* [17]).

The acid catalysis required for the cyclization of the secodopas **2** and **3** corroborates the needs of imine formation; a 'bell-shaped' reaction-rate profile is generally observed in the formation of imines for which the optimum is near pH 4 [18]. In principle, the spontaneous cyclization of **2** and **3** could have led to two products following the addition of the amino group to one or the other of the two carbonyl C-atoms of the conjugated system. Significant is the exclusive preference for the carbonyl C-atom neighboring the carboxylate group which appears to be related to the stability of the corresponding cyclized products.

The cyclization, obviously initiated by the nucleophilic addition of the free amino group to a carbonyl C-atom, or to a vinylogous position, depends on both the acid-base equilibria of the ammonium and of the enol function. Probably there is a relation between the pH range for optimal cyclization rates of **2** and **3** and the pK 's of the enolic system.

Whereas many examples of epimeric betacyanins (as *e.g.* betanin (**8**) and isobetanin) are known, betaxanthins appear as unique species in nature. This is the first time that isoindicaxanthin is characterized analytically; in HPLC, it precedes indicaxanthin (**7**) contrasting thus to the case of the known isomeric betacyanins [19] where the betacyanin always elutes earlier than the isobetacyanin.

The fact that, enzymatically, from the natural L-dopa, pure (*S*)-betalamic acid (**4**) is produced makes it unlikely that naturally produced betalamic acid should not be of pure (*S*)-configuration as well; hence, only betalains of (*S,S*)-configuration would be natural. The origin of the many isobetacyanins isolated from plants [1b] [3a] needs, therefore, to be reexamined. It is known that epimerization of betacyanins to isobetacyanins and *vice versa* takes place easily under alkaline conditions [11] [20]. A possible epimerization in the plant may not be necessarily due to enzymatic action; some general-base catalysis in the aged cell or the conditions of isolation could be responsible for the artificial formation of isobetacyanins.

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Experimental Part

1. *General.* L-Dopa (**1**) was a gift from *F. Hoffmann-La Roche AG*. Betanin (**8**) was isolated from red beet extract by repeated precipitation as Pb-salt following the procedure of [21]. The other products were purchased from *Fluka AG*. Chromatography: *DEAE-Sephadex A-25* and *Sephadex LH-20* from *Pharmacia*, *ODS Hypersyl* from *Shandon*, and *Spherisorb ODS 2* from *Phase Sep*. The centrifugations were realized in a *MSE Multex* centrifuge at 4500 rpm during 10 min at 4°. UV spectra: *Hewlett-Packard-8450A* diode array spectrophotometer.

¹H-NMR spectrum: *Bruker-AC-250* instrument. Buffers: The required molar quantity of salt in H₂O was adjusted to the desired pH's by addition of conc. NaOH soln. or acid (H₃PO₄ or HCl), and the volumes were completed with H₂O; 0.05M NaH₂PO₄ pH 2.8; 0.25M sodium lactate pH 3 and 3.5; 0.25M NaOAc pH 4 to 5; 0.1–0.25M *Tris*-HCl (= [tris(hydroxymethyl)methyl]amine) pH 7.5 and 8; *Tris*-HCl/DTT, 0.1M [tris(hydroxymethyl)methyl]amine + 0.01M dithiotreitol pH 8; MES, 0.25M 2-(morpholino)ethanesulfonate pH 5.5 to 6.5; 0.25M Na₂HPO₄ pH 6.5 and 7; 0.05M Na₂HPO₄/(Bu₄N)₂HPO₄ 0.05M Na₂HPO₄ + 0.03M (Bu₄N)₂HPO₄; 0.25M (NH₄)₂HPO₄ pH 8; (NH₄)₂HPO₄/DTT, 0.1M (NH₄)₂HPO₄ + 0.01M dithiotreitol pH 8; (NH₄)₂HPO₄/DTT/glyc, 0.1M (NH₄)₂HPO₄ pH 8 + 0.01M dithiotreitol + 50% glycerol.

2. *HPLC Chromatography and Quantitative Evaluation of Products.* We used a *Spectra-Physics-SP8700* solvent-delivery system and for detection a *Hewlett-Packard-1040A* diode array spectrophotometer with data processing by a *HP-85B* computer. The columns filled by our own means (25 × 0.5 cm) were protected by a prefiltration cartridge (2 × 0.5 cm); the solvent flow was generally fixed to 0.9 ml/min. *L*-Dopa (**1**), betalamalic acid (**4**), muscaflavin (**5**), 4,5- and 2,3-secodopa (**2** and **3**, resp.) were separated on *ODS Hypersyl* (5 μ) using Na₂HPO₄/(Bu₄N)₂HPO₄ pH 6, with a 0–40% MeOH gradient within 25 min, and then isocratic for 10 min. Betalains (betacyanins and betaxanthins) were separated on *Spherisorb ODS 2* (5 μ) with 0.05M NaH₂PO₄ pH 2.8 [19a] isocratic for 10 min, then with a 0–35% MeOH gradient within 25 min; for optimal separation of indicaxanthin (**7**)/isindicaxanthin these conditions were slightly modified (isocratic for 5 min, then 0–20% MeOH gradient within 30 min). The absorbance at the selected wavelength was recorded through a 0.6-cm flow cell, and separated peaks were integrated yielding 'mAUs' ('milli absorbance units second'), the product of absorbance axis and time axis. Dividing 'mAUs' by 1000, multiplying by 0.9/60 (the solvent flow in ml/s), and dividing by the injected sample volume (inj. vol. in ml) gives the absorbance (*A*) of the product contained in the peak dissolved in 1 ml of soln. The molar concentration is then obtained from the *Beer-Lambert* equation: [mmol/ml] = 'mAUs' · 10⁻³ · [(0.9/60) · 0.6] · (1/ε) · (1/vol. inj.).

3. *Enzymatic Assays.* 3.1. *Enzyme Preparation.* The red skin peeled from caps of the fly agaric *Amanita muscaria* was dropped into liq. N₂ and conserved frozen at –30°. Before extraction, the slices were ground under liq. N₂ in a mortar. The powder obtained was suspended for a few min in (NH₄)₂HPO₄/DTT pH 8 at 4° (18 ml per 4 g of peel), and the soln. was centrifuged. The pellet was resuspended in some (NH₄)₂HPO₄/DTT pH 8 (8 ml) and again centrifuged. To the combined supernatant (22 ml), acetone (9 ml; –30°) was added (*i.e.* 30% of total vol.), and the precipitate was centrifuged; with addition of more acetone (24 ml; completing to 60% of total vol.), another precipitate, containing the metapyrocatechase activity, was formed and centrifuged. The pellet was dissolved in (NH₄)₂HPO₄/DTT pH 8 (6 ml), and acetone (9 ml; 60% of total volume) was added to precipitate the proteins which were centrifuged. This pellet was dissolved in buffer or H₂O at 4° for enzymatic assays.

3.2. *Substrate Specificity of the Metapyrocatechase.* In a temperature-controlled UV cell, substrate (0.3 ml, 0.01M), (NH₄)₂HPO₄ pH 8 (0.6 ml, 0.25M), H₂O (2 ml), and enzyme preparation (0.1 ml of an extract from 2 g of tissue dissolved in 5 ml of (NH₄)₂HPO₄/DTT/glyc pH 8) were mixed at 25° (conc. of substrate in the assay 0.001M). The development of the yellow solns. was followed by measuring the absorbance at λ_{max} for a period of 20 min during which the increase remained linear. The absorbance after 10 min and, in parentheses, λ_{max} [nm] of the

Table 3. *Tests of Residual Activity of Enzymic Solutions after Storage at Different Temperatures.*
Absorbance (*A*) at 420 nm after 30 min incubation with **1** at 25° and pH 8.

Time [min]	Temp. 4°		Temp. 25°		Temp. 37°	
	<i>A</i>	<i>A</i> _{rel}	<i>A</i>	<i>A</i> _{rel}	<i>A</i>	<i>A</i> _{rel}
0	0.87	1.00	0.87	1.00	0.87	1.00
10	0.89	1.02	0.90	1.03	0.70	0.80
20	0.92	1.06	0.85	0.98	0.52	0.60
40	0.89	1.02	0.74	0.85	0.33	0.38
60	0.88	1.01	0.76	0.87	0.21	0.24
90	0.94	1.08	0.74	0.85	0.14	0.16
120	0.90	1.03	0.67	0.77	0.06	0.07
180	0.86	0.99	0.59	0.67	0.05	0.06
240	0.84	0.97	0.58	0.67	0	0
360	0.84	0.97	0.47	0.54	0	0
1320	0.71	0.82	0	0	0	0

products and the absorbance relative to that obtained in the test with L-dopa are: L-dopa 0.119 (416, rel. 1.0); D-dopa 0.074 (416, rel. 0.62); α -methyl-L-dopa 0.133 (434, rel. 1.12); dopamine 0.201 (426, rel. 1.69); *N*-acetyl-dopamine 0.015 (384, rel. 0.13); pyrocatechol 0.023 (377, rel. 0.19); (3,4-dihydroxyphenyl)acetic acid 0.005 (382, rel. 0.04). The following substrates did not react: 3-(3',4'-dihydroxyphenyl)propionic acid, caffeic acid, 3,4-dihydroxybenzaldehyde, and 3,4-dihydroxybenzoic acid.

3.3. *Thermostability of the Enzymic System.* Samples (1 ml) of enzyme soln. (prepared from 3.1 g of tissue and diluted in 4 ml of $(\text{NH}_4)_2\text{HPO}_4$ /DTT pH 8) were left at 4, 25, and 37°. The enzymatic activity of these solns. was tested at regular intervals by assays (0.1 ml) incubated at 25° with L-dopa (1; 0.1 ml, 0.01M), H_2O (0.6 ml), and $(\text{NH}_4)_2\text{HPO}_4$ pH 8.0 (0.2 ml, 0.25M). After 30 min, MeOH (1.5 ml) was added to precipitate the proteins which were removed by centrifugation before measuring the absorbance of the supernatant at 420 nm (Table 3).

4. *Identification of the Products Enzymatically Generated from L-Dopa.* 4.1. *Betalamic Acid (4) from Betanin (8).* Conc. NH_3 was added under N_2 to a suspension of 4–5 mg of 8 in H_2O to achieve complete dissolution at pH 12.5. The soln. remained 45 min at 25° under N_2 , then the pH was adjusted to 11.5 and the mixture chromatographed on DEAE-Sephadex A-25 (25 $\times$ 2 cm, Cl^- form; 0.3–1M NaCl gradient) [12]. The purity of the fractions of the yellow zone collected was checked by HPLC: 4 at t_R 27 min; λ_{max} 430 nm.

4.2. *Isolation of Muscaflavin (5) Extracted from the Cap of the Fly Agaric.* Powdered red peel of *Amanita muscaria* (3.5 g) was extracted at 4° with MeOH (12 ml). The mixture was sonicated for 30 s and centrifuged. The pellet was once more extracted and centrifuged. The extracts were evaporated and the residue, dissolved in 0.3M NaCl (4 ml), was chromatographed on DEAE-Sephadex A-25 (25 $\times$ 2 cm, Cl^- form; 0.3–1M NaCl gradient, flow rate 44 ml/h) [12]. The absorbance of the eluted fractions (7.3 ml) was measured at 420, 480, and 540 nm (Fig. 2). The zone containing 5 eluted together with a red pigment (Fr. 38–44, in Fig. 2). Fr. 39, examined by HPLC (0.1 ml), contained practically pure 4 (t_R 27 min; λ_{max} 430 nm) and Fr. 43 practically pure 5 (t_R 30.5 min; λ_{max} 422 nm). The latter was concentrated under vacuum at 30° to 1 ml and stored at –30° to serve as HPLC standard.

4.3. *Identification of Enzymatically Produced Betalamic Acid (4) and Muscaflavin (5).* L-Dopa (1, 0.1 ml, 0.01M) was added to Na_2HPO_4 pH 6.5 (0.2 ml, 0.25M), H_2O (0.6 ml), and enzyme preparation (0.1 ml of an extract from 4 g of tissue and diluted in 3 ml of $(\text{NH}_4)_2\text{HPO}_4$ /DTT pH 8) and incubated 4 days at 4°. HPLC (detection at 420 nm) of the yellow soln. t_R 18.5, 21.5, 27, and 30.5 min (Fig. 1). The peak at t_R 27 min was identical with that of 4 prepared from 8 (coinjection: 1 peak of added surfaces). The peak at t_R 30.5 min was identical with that of 5 isolated from *Amanita* [6a] (coinjection 1 peak of added surfaces).

4.4. *Correlation of 4,5-Secodopa (2) and 2,3-Secodopa (3) with 4 and 5, Respectively.* The mixture of yellow compounds generated according to 4.3 was separated by HPLC. The zones at t_R 18.5 (2.9 ml; 3) and 21.5 min (1.6 ml; 2) were concentrated under vacuum at 30° and adjusted to the same volumes (0.15 ml). NaOAc buffer pH 5.5 (0.075 ml, 1M) was added to each sample. After 14 h at 4°, the products were examined by HPLC: the soln. obtained from 3 showed 1 product (t_R 30.5 min, λ_{max} 422 nm), identical and coincident with 5, and the soln. obtained from 2 contained also 1 product (t_R 27 min, λ_{max} 430 nm), identical with 4.

5. *Enzymatic Cleavage Products from L-Dopa (1).* 5.1. *Enzymatic Incubation of 1 at Various pH's.* A mixture of 0.01M aq 1 (0.2 ml) buffer (0.4 ml, 0.25M), H_2O (1.2 ml), and enzyme preparation (0.2 ml of an extract from 8 g of tissue and diluted in 10 ml of $(\text{NH}_4)_2\text{HPO}_4$ /DDT/glyc pH 8) was incubated at 4° (buffers: MES pH 6.5; Na_2HPO_4 pH 7; $(\text{NH}_4)_2\text{HPO}_4$ pH 7.5 and 8); final concentrations were 0.001M 1 and 0.05M buffer. Daily samples (0.08 ml) were taken and stored in the freezer (–30°); of the thawed samples, 0.035 ml were analyzed by HPLC at 280 (for 1) and 420 nm (for 2–5): see Table 4.

Table 4. *Product Development in Enzymatic Assays with 1 at Different pH's (see Fig. 3).*

The values in the table are 'mAU's' (integrated areas of HPLC peaks); from these, molar concentrations can be calculated by the conversion factors.

Time [day]	1 0.255 $\cdot 10^{-6}$ a)	2 0.028 $\cdot 10^{-6}$ a)	3 0.028 $\cdot 10^{-6}$ a)	4 0.0238 $\cdot 10^{-6}$ a)	5 0.0238 $\cdot 10^{-6}$ a)
pH 6.5					
0	3453	0	0	0	0
1	3091	777	2048	740	0
2	2688	846	3525	1917	0
3	2383	657	4161	2997	552
4	2116	660	4522	3893	802

Table 4 (cont.)

Time [day]	1 0.255 · 10 ^{-6a})	2 0.028 · 10 ^{-6a})	3 0.028 · 10 ^{-6a})	4 0.0238 · 10 ^{-6a})	5 0.0238 · 10 ^{-6a})
5	1946	543	4529	4493	1051
6	1790	435	4296	4918	1292
7	1718	366	4106	5442	1591
8	1571	0	3465	5236	1720
9	1581	0	3144	5487	1943
10	1370	0	2465	5544	1974
pH 7					
0	3453	0	0	0	0
1	2440	1592	4074	866	0
2	1979	1843	6283	2486	0
3	1583	1579	7081	3484	346
4	1470	1399	7491	4717	617
5	1453	1077	7266	5352	776
6	1355	806	6474	5770	935
7	1347	618	5994	5940	1078
8	673	355	4970	5665	1144
9	670	0	4518	5810	1276
10	468	0	3954	5833	1299
pH 7.5					
0	3453	0	0	0	0
1	1818	3704	8209	1278	0
2	1250	4211	10726	2686	0
3	985	3541	10339	3796	0
4	935	3016	9670	4736	465
5	542	2329	8820	5434	563
6	523	1765	7829	5887	665
7	311	1327	6576	6272	909
8	153	913	5312	5884	914
9	0	674	4755	6198	900
10	0	439	3564	6071	891
pH 8					
0	3453	0	0	0	0
1	1332	4987	11291	1471	0
2	773	5093	12719	2672	0
3	548	4456	11961	3562	0
4	334	3733	11103	4470	423
5	283	2977	9966	5153	497
6	193	2293	8766	5686	745
7	0	1775	7693	6012	804
8	0	1230	6145	5793	827
9	0	881	5382	6023	767
10	0	592	4048	5918	800

a) Conversion factor.

5.2. *Evaluation of an ε -Value for the Secodopas 2 and 3.* The sum of the molar concentration of 2 and 3, obtained according to 5.1, was calculated as the difference of enzymatically converted 1 minus 4 and 5 (see Table 4), based on their known molar absorptivities (ε (1) = 2800, ε (4 or 5) = 30 000). The ε value of secodopa are calculated for each anal. set; they decrease linearly with the time of the analysis (probably a consequence of products depletion), and the slope is nearly identical in all four series of data sets; extrapolation to zero time converges in all the series to a value ε 25 500 $\pm$ 500.

5.3. *Transformation of Secodopas 2 and 3 at Different pH's.* A mixture of 2/3 was generated by incubation of enzyme soln. (5 ml; see 5.1), **1** (98.5 mg, 0.05 mmol), $(\text{NH}_4)_2\text{HPO}_4$ pH 8 (5ml, 2M), and H_2O (90 ml) for 3 days at 4°. Aliquots (0.3 ml) of the resulting yellow soln. were transferred at 4° to 0.25M buffers (1.2 ml) of different pH's; sodium lactate pH's 3, 3.5; NaOAc pH's 4, 4.5, 5; MES pH's 5.5, 6, 6.5. The development of the product mixtures was followed for 12 h by HPLC of samples (0.06 ml) taken at certain time intervals (see Table 5).

Table 5. *Changes of Product Compositions after Incubation of an Enzymatically Generated Mixture of Secodopas 2 and 3 at Different pH's.* Substance amounts ('*MAUs*'), determined after 2 h, are converted to molar concentrations by the conversion factors (see Fig. 4).

Time [h]	2 $0.0163 \cdot 10^{-6a)}$	3 $0.0163 \cdot 10^{-6a)}$	4 $0.0139 \cdot 10^{-6a)}$	5 $0.0139 \cdot 10^{-6a)}$	Dopaxanthin $0.001 \cdot 10^{-6a)}$
pH 3					
0	4157	8716	1796	0	366
2	2969	6203	2266	1138	348
5	2341	5010	2267	1532	320
12	1490	2573	2109	2649	364
pH 3.5					
0	4157	8716	1796	0	366
2	2776	5910	2404	1220	339
5	1462	4270	2049	1674	342
12	1149	1907	2388	2885	371
pH 4					
0	4157	8716	1796	0	366
2	2398	4943	2738	1497	347
5	1294	1880	2781	2542	363
12	667	941	3106	3628	424
pH 4.5					
0	4157	8716	1796	0	366
2	2065	3635	2966	1755	330
5	898	666	3311	2937	359
12	398	329	3835	4014	330
pH 5					
0	4157	8716	1796	0	366
2	2033	2960	2808	1335	360
5	800	1024	3274	2282	392
12	traces	traces	3775	3236	459
pH 5.5					
0	4157	8716	1796	0	366
2	2613	5225	2778	743	354
5	2049	4717	2948	1195	971
12	1147	3165	4039	2037	915
pH 6					
0	4157	8716	1796	0	366
2	3013	7074	2664	388	374
5	2399	6273	3066	687	682
12	1522	4079	3421	1146	1178
pH 6.5					
0	4157	8716	1796	0	366
2	4180	8211	1327	0	252
5	2914	6646	2412	444	519
12	2235	7303	3223	559	743

^{a)} Conversion factor.

6. *Formation of Betalains*. 6.1. *Enzymatic Assay with L-Proline in Tris and Hydrogen Phosphate Buffer*. Two assays were incubated at 4° with **1** (0.1 ml, 0.01M), L-proline (0.05 ml, 0.05M), H₂O (0.55 ml), enzyme preparation (0.1 ml of an extract from 4.3 g of *Amanita* dissolved in 1.5 ml of H₂O), and buffer (0.2 ml, 0.25M; i.e. Tris-HCl pH 7.5 and Na₂HPO₄ pH 7.5, resp.). The formation of *Tris*-betalain and indicaxanthin (**7**) was followed by HPLC at 480 nm (conditions for betalains) of aliquots (0.035 ml) injected after different incubation times (Table 6).

Table 6. Amounts (expressed as 'mAU's') of Betaxanthins Formed in Enzymatic Assays with L-Dopa (**1**) and L-Proline

	Time [day]					
	1	4	7	11	17	24
<i>Tris</i> -HCl buffer						
<i>Tris</i> -betalain	592	2617	2672	1871	1308	779
Indicaxanthin (7)	510	4947	10125	16468	20676	24151
Na ₂ HPO ₄ buffer						
<i>Tris</i> -betalain	0	0	0	0	0	0
Indicaxanthin (7)	536	5749	11129	16196	20058	23122

6.2. *Preparation of Indicaxanthin (7) from Betanin (8)*. To a soln. of **8**/isobetanin (3.6 mg, 0.067 mmol) in 17% NH₃/H₂O (2 ml), L-proline (7.7 mg, 0.067 mmol) was added. The mixture was left 1 h at 25°, then acidified with HCl to pH 1, and submitted to HPLC: *t*_R 25.7 (4985 'mAU's', 65%; **7**) and 25.2 min (2688 'mAU's', 35%; isoindicaxanthin). HPLC of starting material (conditions for betalains): *t*_R 19.8 (8789 'mAU's' 67%; **8**) and 21 min (4355 'mAU's', 33%; isobetanin).

6.3. *Identification of Enzymatically Produced 7*. L-Dopa (**1**; 0.1 ml, 0.01M), *Tris*-HCl pH 7.5 (0.2 ml, 0.25M), L-proline (0.5 ml, 0.02M), and enzyme preparation (0.1 ml of an extract from 2.3 g of *Amanita* and dissolved in 1 ml H₂O) were incubated 7 days at 4°. HPLC (conditions for betalains): only one peak *t*_R 25.5 (**7**); coinjection of enzymatically prepared **7** and synthetic **7**/isoindicaxanthin (see 6.2) gave 2 peaks at *t*_R 25.1 and 25.6 min, the latter being greatly enhanced.

6.4. *Enzymatic Syntheses of Indicaxanthin (7) and Isoindicaxanthin*. Dopa (0.1 ml, 0.01M), *Tris*-HCl buffer pH 7.5 (0.05 ml, 1M), H₂O (0.7 ml), L-proline (0.05 ml, 0.2M), enzyme preparation (0.1 ml of an extract from 4.9 g of *Amanita* and dissolved in 1 ml of *Tris*-HCl/DTT pH 8) was incubated 3 days at 4°. Three such enzymatic assays were prepared identically with L-, D-, and DL-dopa as substrates, indexed as 'L', 'D', 'DL', resp. Identical aliquots of 'L', 'D', and 'DL' (0.035 ml each) and 1:1 mixtures (0.035 ml + 0.035 ml) 'L'/'DL' and 'D'/'DL' were analyzed by HPLC under modified conditions (*Spherisorb ODS 2* (5 μ) with 0.05M NaH₂PO₄ pH 2.8 [19a] isocratic for 5 min, then 0–20% MeOH gradient within 30 min) for optimal separation of a mixture of **7** (*t*_R 24.8 ± 0.1 min) and isoindicaxanthin (*t*_R 23.9 ± 0.1 min); the peak of *Tris*-betalain appears at *t*_R 9.6 min. Results (peak integrations in 'mAU's'): 'D': 2661 (100%, isoindicaxanthin); 'L': 2648 (100%, **7**); 'DL': 1194 (42%, isoindicaxanthin); 1635 (58%, **7**); 'D'/'DL': 4359 (67.3%, isoindicaxanthin), 2978 (32.3%, **7**); 'L'/'DL': 1418 (21.4%, isoindicaxanthin), 5214 (78.6%, **7**).

7. *Preparative Enzymatic Formation of Muscaflavin (= 4-Formyl-2,3-dihydro-1H-azepin-2,7-dicarboxylate; 5)⁸*. A mixture of **1** (99 mg, 0.5 mmol), (NH₄)₂HPO₄ (2.5 ml, 2M, pH 8), and 2.5 ml of enzyme extract (from 6 g of tissue of *Amanita muscaria*, adjusted to 50 ml with H₂O) was incubated during 6 days at 4°. The soln. was acidified to pH 5.5 by adding AcOH and left 24 h at 4°. The pH was again increased to 7.5, and 287 mg of L-proline (2.5 mmol) were added. After 3 days at 4°, the soln. was acidified with conc. HCl soln. to pH 1.5 and the aq. phase extracted 4 times with 100 ml of AcOEt. The combined org. phase was dried (MgSO₄) and evaporated. The residue (32 mg) was chromatographed on *Sephadex LH-20* (23 × 2.2 cm, MeOH). The fractions containing pure **5** (HPLC) yielded a brownish residue (2 mg) on evaporation. ¹H-NMR (CD₃OD, 250 MHz): 9.38 (s, 1 H, CHO); 7.16 (d, *J* = 8.5, 0.85 H, H-C(5)); 7.16 (s, 0.1–0.15 H, H-C(5), H/D exchange of H-C(6) with solvent); 6.20 (d, *J* = 8.5, 0.85 H, H-C(6), H/D exchange with solvent); 4.28 (dd, *J* = 6.5, 1.5, 1 H, H-C(2)); 3.33 (dd, *J* = 15, 1.5, 1 H, H-C(3)); 2.93 (dd, *J* = 15, 6.5, 1 H, H-C(3)).

8. *Spectrometric Titration of Enzymatically Generated Solutions of the Secodopas and of the Cleavage Product from Pyrocatechol*. 8.1. *Enzymatic Assay with 1 in Presence of Pyrocatechol*. To *Tris*-HCl pH 8 (0.2 ml, 0.25M),

⁸) In collaboration with Philippe Hünenberger.

1 (0.1 ml, 0.01M), and enzyme preparation (0.1 ml of an extract from 1 g of *Amanita muscaria* peel and dissolved in 1 ml of 0.1M Tris-HCl pH 8), different volumes of pyrocatechol were added (0.1, 0.3, and 0.6 ml, 0.01M); one assay was left without pyrocatechol as a control experiment. The assays were adjusted with H₂O to the same volume (1 ml). After 3 h incubation at 4°, samples (0.5 ml) were analyzed by HPLC (Table 7).

Table 7. Enzymatic Assay with **1** in Presence of Pyrocatechol.

The product compositions (in 'mAU's') are determined by HPLC after 3 h of incubations; detection at 380 nm for **9** and 420 nm for **2** and **3**.

Starting material		Products		
L-Dopa (1) [M]	Pyrocatechol [M]	Secopyrocatechol (9)	4,5-Secodopa (2)	2,3-Secodopa (3)
0.001	0	—	2209	3571
0.001	0.001	traces	2003	3320
0.001	0.003	857	1772	2987
0.001	0.006	1720	1294	2087

8.2. *UV Spectroscopy of the Secodopas 2 and 3.* By incubation of an enzyme preparation (0.1 ml of an extract from 3 g of *Amanita* dissolved in 0.6 ml of 0.1M of Tris-HCl/DTT pH 8), H₂O (1.8 ml), and **1** (0.1 ml, 0.01M) for 3.5 h at 4°, **2/3** was generated (conserved frozen for 12 days without notable change) and separated by HPLC in 4 runs of 0.1 ml each. The fractions containing **3** (10 ml) or **2** (7.2 ml) were pooled and concentrated under vacuum at 25° to 3.1 and 3.4 ml, resp. For the UV/VIS spectra, 0.2 ml of these solns. and 0.6 ml of different 0.25M buffers were mixed. The stability of **2** and **3** at different pH's was checked by recording spectra every 30 s during 5 min. The pH of the solns. was measured after UV recordings. Buffers: sodium lactate pH 3; NaOAc pH's 4, 4.5, and 5; MES pH's 5.5, 6, and 6.5; Na₂HPO₄ pH 7; Tris-HCl pH's 7.5 and 8 (Fig. 5).

8.3. *UV Spectroscopy of Secopyrocatechol (9).* Pyrocatechol (0.1 ml, 0.01M), H₂O (1.7 ml), and enzyme preparation (0.2 ml of an extract from 3 g of *Amanita* peel dissolved in 0.6 ml of 0.1M Tris-HCl/DTT pH 8) were incubated at 4° for 3.5 h. The mixture was deproteinated by addition of 3 ml of acetone followed by centrifugation. The yellow supernatant containing **9** was concentrated under vacuum at 25° to ca. 0.3 ml and adjusted with H₂O to 1.1 ml. For the spectrometric titration, 0.9 ml of 0.25M buffer and 0.1 ml soln. of **9** were mixed as described in 8.2 (Table 8). The linearization method for pK_a calculations was based on the formula $(OD - OD(B)) \cdot 10^{pH} = (-1/K_a) \cdot OD + (1/K_a) \cdot OD(BH)$ [22]. The value OD(B) is the absorption at the 'alkaline end' of titration; the value OD(BH) is an uncertain constant ('acid end' of titration) but does not interfere in the calculation of the constant K_a. The value pK_a is given from 1/K_a, value which corresponds to the slope of the line of points (calculated by the method of least squares) defined by the coordinates $(OD - OD(B)) \cdot 10^{pH}$, and OD.

Table 8. Spectrometric Titrations of Secocatechols (based on the curves in Fig. 5).

Absorptions (OD) at the wavelength of the maximas corresponding to BH and B at various pH.

4,5-Secodopa (2)			2,3-Secodopa (3)			Secopyrocatechol (9)		
pH	OD (380)	OD (424)	pH	OD (372)	OD (414)	pH	OD (322)	OD (378)
3.1 ^{a)}	0.034	0.034	3.2	0.068	0.028	3.0 ^{a)}	0.244	0.050
4.0	0.027	0.043	4.05	0.056	0.040	5.0	0.23	0.096
4.65	0.026	0.049	4.7	0.049	0.054	5.5	0.211	0.108
4.95	0.025	0.053	5.0	0.042	0.067	5.9	0.201	0.145
5.4	0.025	0.059	5.45	0.032	0.081	6.4	0.167	0.205
5.8 ^{a)}	0.022	0.060	5.8 ^{a)}	0.027	0.089	7.0	0.108	0.324
6.8	0.022	0.064	6.8	0.016	0.100	7.4 ^{a)}	0.070	0.361
						8.0	0.070	0.446
pK _a 4.82			pK _a 4.98			pK _a 6.72		

^{a)} Values neglected for the calculation of pK_a.

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Review

Amanita muscaria: chemistry, biology, toxicology, and ethnomycology

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The fly agaric is a remarkable mushroom in many respects; these are its bearing, history, chemical components and the poisoning that it provokes when consumed. The 'pantherina' poisoning syndrome is characterized by central nervous system dysfunction. The main species responsible are *Amanita muscaria* and *A. pantherina* (Amanitaceae); however, some other species of the genus have been suspected for similar actions. Ibotenic acid and muscimol are the active components, and probably, some other substances detected in the latter species participate in the psychotropic effects. The use of the mushroom started in ancient times and is connected with mysticism. Current knowledge on the chemistry, toxicology, and biology relating to this mushroom is reviewed, together with distinctive features concerning this unique species.

INTRODUCTION

The fly agaric, *Amanita muscaria*, and the panther, *A. pantherina*, are the species mainly involved in the 'pantherina-muscaria' poisoning syndrome. Poisoning cases are sometimes accidental and mainly those caused by *A. pantherina*, since this mushroom might be mistaken for some other species; but, in some cases, intentional consumption of *A. muscaria* occurs for recreational purposes. Prognosis of the poisoning is generally minor; although, very seldom lethal cases are mentioned. Central nervous system dysfunctions primarily characterize this poisoning. This review describes the unusual features associated with these species, medical, chemical, pharmacological, historical and phytogeographical and aims to synthesize current knowledge on these matters. The emphasis is, however, the chemical and biological properties of the substances found in the mushroom.

THE SUSPICIOUS SPECIES

Detailed mycological data have already been published (Moser 1983, Bresinsky & Besl 1990, Takashi, Chihiro & Mitsuya 1999, Neville & Poumarat 2001). The main responsible species are *Amanita muscaria* (Fig. 1) and *A. pantherina*. The cap of *A. muscaria* can be

50 cm diam and bright red, orange, or even orange or yellow, apart from the white fleck. Many species of the *A. muscaria* complex bear so-called crassosporos (Tulloss & Halling 1997). The species cannot be mistaken for any other perhaps except the edible *A. caesarea*. *A. pantherina* is also known as: 'panther cap', 'panther Amanita', 'panther agaric' (North America), 'pantherschwamm', 'krötenschwamm' (Germany), 'tignosa' (Italy), 'pantera', 'pixaca', 'hongo loco', 'hongo malo' (Spanish America) and 'amanite panthère' (French). The cap of *A. pantherina* is 5–10 cm diam and grey or grey-brown, grey yellow, paling when old, with small pure white flakes. This species, in some accidental poisoning cases, has been mistaken for the edible *A. rubescens* and *A. spissa*. These two species occur in many continents (Neville & Poumarat 2001) and grow in deciduous woods, especially beech and birch, and also coniferous ones (Hotson & Lewis 1934).

Some related species are suspected of poisoning or produce the active components detected in these fungi (Bresinsky & Besl 1990): *A. regalis* (Elonen, Tarssanen & Härkönen 1979) and *A. strobiliformis* (Takemoto, Yokobe & Nakajima 1964; syn. *A. solitaria* auct.). A few cases of poisoning by *A. gemmata* (Cornué 1961; syn. *A. junquillea*), *A. crenulata* (Buck 1965, Tulloss 1990), and *A. cothurnata* have also been reported, but their possible toxic potencies are disputed (Chilton & Ott 1976). Toxin content has been suggested to be related to the place of gathering, indicating chemical

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Fig. 1. *Amanita muscaria* (Italy: Piemonte, in woodland south of Torino, 1998). Photo: D. L. Hawksworth.

intraspecific variation (Benedict, Tyler & Brady 1966). Surprisingly, Chilton & Ott (1976) mentioned large amounts of chlorocrotylglycine and amino-2-hexadien-4,5-oic acid, both derivatives of glycine, in *A. smithiana*. Noting the functional identity ('toxicophore') of these latter substances, an enzyme inhibitor activity has to be suspected; nephrotoxic poisonings by this species have been reported (Leathem *et al.* 1997).

THE POISONING SYNDROME

Characteristics of poisoning and symptomatology

The poisoning syndrome due to *Amanita muscaria* and *A. pantherina* has been called 'mycoatropinic', as the symptoms are similar to those induced by atropinic plants such as *Datura stramonium*, *Atropa belladonna*, and *Hyoscyamus niger*, but tropanic alkaloids are not present.

Chronosymptomatology is variable amongst subjects, depending on the rituals (Waser 1967, Festi 1985), and experimental and accidental poisonings. In rituals, the poisoned person (e.g. the shaman) is in search of a peculiar state of mind where autosuggestion is important. A 'delirium' is not completely created but the chemicals enhance the intellect (Levi-Strauss 1970).

Socio-cultural context and environment associated with the psychological and physiological state of the participant are pertinent (Rosenbohm 1995).

The 'pantherina-muscaria' syndrome is atropine-like and in the number and severity of poisoning cases fatality is rare. In most cases recovery is virtually complete after 24 h without noticeable after-effects (Bosman *et al.* 1965, Gerault & Girre 1977, Hatfield 1979, Lampe 1979, Bornet 1980, Gelfand & Harris 1982, Pegler & Watling 1982, Iancovitz 1983, Hanrahan & Gordon 1984, Benjamin 1992, Denoyer 1992). In contrast to *Cortinarius* (Michelot & Tebbett 1990), or *Gyromitra* poisoning syndromes (Michelot & Toth 1991), the fly agaric effects have a short latency like the *Coprinus* syndrome (Michelot 1992). In most cases as little as one cap, a cup of sautéed mushrooms, is a sufficient for psychotropic effects.

Symptoms start 30 min to 2 h after ingestion. A state of confusion, dizziness, and tiredness, visual and auditory aesthesia (hypersensitivity), space distortion, and unawareness of time; presents reinforced by the consumption of some psycho-sedative agents. Aggressive attitudes have not been reported. Dryness of the mouth and mydriasis (dilation of the pupils) have been mentioned. Hallucinations, vivid colour perception and a sense of time standing still, are disputed. A drowsiness period after 2 h follows, with vivid dreams. A deep sleep ends the poisoning, which lasts generally 8 h (Benjamin 1992, Davis & Williams 1999).

Subsequent gastrointestinal disorders with vomiting are inconstantly reported and are not characteristic of the syndrome (Festi & Bianchi 1992). No damage to organs has been reported although the active components may induce *in vivo* brain lesions. Regular consumption of the mushroom would probably be harmful, even though most human poisoning cases do not report any after-effects. Brain lesions in rodents treated with ibotenic acid and muscimol can occur (Lescaudron, Bitran & Stein 1992). According to Festi & Bianchi (1992), the biological effects are related to the period of collection, those collected in September induce more marked nausea and less narcotic and visionary experience than those collected in August. Mushroom intoxication in dogs has been reported; the chronosymptomatology is consistent with that in humans (Naudé & Berry 1997).

The symptomatology and chronology of poisoning events are summarized in Fig. 2.

Treatment

The appropriate treatment is mainly symptomatic. First, eliminate the toxic substances from the gastrointestinal tract out by vomiting (e.g. administration of ipecac syrup; Benjamin 1992), stomach washout, or the administration of activated charcoal and purging, preferably in an intensive care emergency unit (M. Denoyer, pers. comm.). The actions of the active principles, ibotenic acid and muscimol, have to be

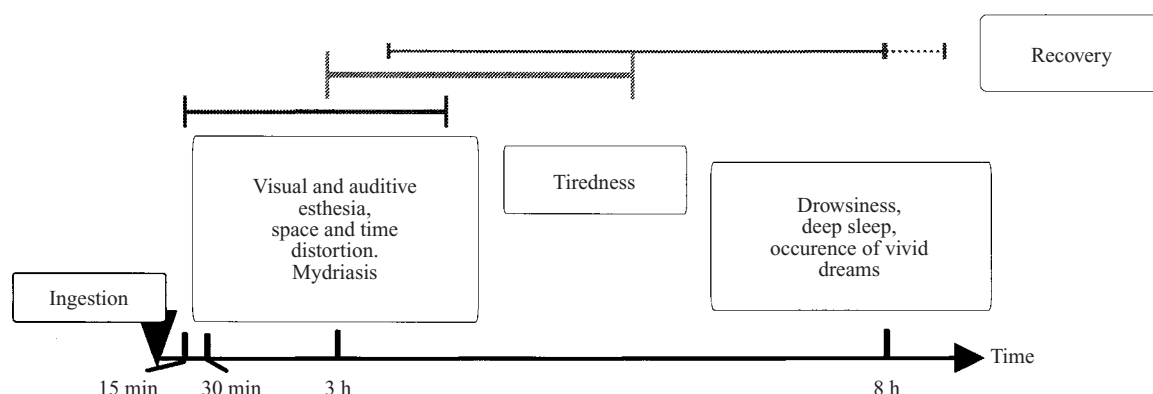


Fig. 2. Chronosymptomatology of the *Amanita muscaria* poisoning syndrome.

compared with that of atropine; consequently the use of the latter drug is strongly contraindicated. Physostigmine (eserine), a cholinesterase inhibitor, has been recommended as it counteracts the effects of poisoning by atropine and related antimuscarinic drugs. The intravenous dosage for adults and adolescents is 1–2 mg repeated when needed (Taylor 1992), the drug has also been used successfully in veterinary cases (Ridgway 1978). The administration of sedatives such as diazepam or clonazepam, by mouth or intravenously, in case of convulsions, as well as phenobarbitone has been suggested (Lambert & Larcen 1989, Garnier, Azoyan & Baud 1990, Benjamin 1992, Denoyer 1992). However, diazepam has been suspected as strengthening the action of muscimol (Hanrahan & Gordon 1984, Benjamin 1992). Contrary to some statements, cooking does not notably lower toxicity, demonstrating that the active components are not heat sensitive.

Legal status and enforcement

About 12 species of mushrooms are consumed for recreational purpose, but seldom *Amanita muscaria* (Samorini 1992). The Italian courts proscribe the use of psilocybin, psilocin and *A. muscaria* as well ('Stampa alternativa'). However, in Europe, interest in this type of drug is lower than in North America, where laws prohibit its use. The use of *A. muscaria*, even in Europe, is nevertheless growing. In some countries, possession of hallucinogenic mushrooms is not illegal, contrary to their purified substances and derivatives. In Europe, the current legislation mainly concerns *Psilocybe semilanceata*, and to a lesser extent *A. muscaria* (Festi 1985).

CHEMICAL PRODUCTS

The fly agaric represented a challenge to chemists, biochemists, and biologists. The goals were: (1) the detection and mode of action of the substances involved in the psychotropic effect that might ultimately lead to the design of new drugs; (2) the isolation and chemical identification of the pigments responsible for

the characteristic hue of the cap; and (3) the discovery of miscellaneous unusual components. Amazingly, most substances produced are chemically and biogenetically interrelated, a common molecular architecture linking most of the structures (Eugster 1969).

The true toxins

Although the chemical constituents of this mushroom have been thoroughly investigated, a few details remain unclear; they concern the relationships between the physicochemical properties of the active components and the mode of consumption. For instance, Mexican people eat the carpophore of *Amanita muscaria* without the cuticle that has been peeled off, and also discard the cooking water (Pérez-Silva & Herrera-Suarez 1991). In Italy, after boiling and rejecting excess water, the mushroom is preserved in brine prior to consumption (Festi 1985). In North America, the red cuticle is peeled off, and the remainder dried, and then smoked (Ott 1978). These latter procedures would eliminate or destroy the greater part of the active water-soluble substances.

Muscimol and ibotenic acid are most likely to be the biologically active principles of the species of concern, but other active components are suspected. Different research groups performed almost simultaneous isolation and structure determination of the active components (Takemoto & Nakajima 1964c, Bowden & Drysdale 1965, Eugster, Müller & Good 1965, Good, Müller & Eugster 1965). The substances are isoxazole derivatives: ibotenic acid **1** (from 'iboten-gu-take', the Japanese name of *A. strobiliformis*, previously called 'pre-muscimol'; Good *et al.* 1965, Eugster & Takemoto 1967), its decarboxilation gives muscimol **2**; these compounds are only reported in *A. muscaria* and *A. pantherina* subspecies (Benedict *et al.* 1966).

Ibotenic acid (pantherin, agarin), i.e. α -amino-3-hydroxy-5-isoxazoloacetic acid **1**, is colourless (crystals, $C_5H_6N_2O_4$, mol wt 158.11, mp 150–152 °C decomposition), readily soluble in cold water. Any attempt of dehydration leads to decarboxilation yielding quantitatively muscimol, thus suggesting that a meal

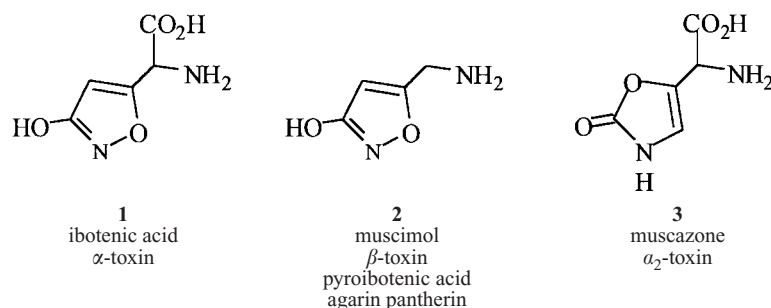


Fig. 3. Chemical structure of ibotenic acid, muscimol and muscazone.

of the cooked mushrooms, or even after gastric digestion, would only contain the latter substance, and be the agent responsible for the main symptoms. The red skin of the cap and the yellow tissue beneath contain the highest amounts of these substances, so explaining practices in removing it (Catalfomo & Eugster 1970a).

Muscimol, i.e. 5-(aminomethyl)-3-hydroxyisoxazole **2**, isolated from *A. muscaria*, is colourless (crystals, $C_4H_6N_2O_2$, mol wt 114.10, mp 175° decomposition) readily soluble in cold water (Bowden & Drysdale 1965). The structure of pantherine, isolated from *A. pantherina*, was found to be similar to that of 'agarin' (Konda, Takahashi & Onda 1985).

Muscazone, i.e. α -amino-2,3-dihydro-2-oxo-5-oxazoloacetic acid **3**, is colourless (crystals, $C_5H_6N_2O_4$, mol wt 158.11, mp $190^\circ C$ decomposition) and was also detected and the structure confirmed by synthesis (Eugster *et al.* 1965, Fritz *et al.* 1965, Reiner & Eugster 1967). This lactame isomer of muscimol would result from photo-rearrangement of the latter structures (Göth *et al.* 1967), and is produced during isolation. Muscazone exhibits minor pharmacological activities in comparison with the previous substances. Interestingly, cycloserine (myxomycin, seromycin, D-4-amino-3-isoxazolidone) is an antimicrobial tuberculostatic agent that exhibits a similar carbon backbone as muscimol. It is known to induce effects on the central nervous system, but with a longer latency period: somnolence, confusion, and nervousness distinguish untoward effects (Mandell & Sande 1992) (Fig. 3).

Several syntheses of ibotenic acid and muscimol have been developed *via* different routes (Gagneux *et al.* 1965a,b, Sirakawa *et al.* 1966, Kishida *et al.* 1966, 1967, Fräter-Schröder, Good & Eugster 1969, Loev, Wilson & Goodman 1970, Nakamura 1971, McCarry & Savard 1981, Jäger & Frey 1982, Welch 1982, Oster & Harris 1983, Frey & Jäger 1985). Thio-derivatives and even more potent inhibitors of GABA uptake have been prepared (Lykkeberg & Krogsgaard-Larsen 1976, Krogsgaard-Larsen 1977). Gram-scale preparation has been performed by regiospecific 1,3-dipolar cycloaddition/elimination (Pevarello & Varasi 1992). Thio-muscimol derivatives have been prepared; they exhibit highly potent binding properties even able to displace GABA from its receptor (Melikian *et al.* 1992).

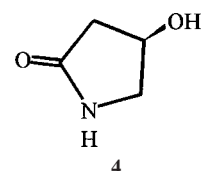


Fig. 4. Chemical structure of the hydroxypyrrolidone derivative.

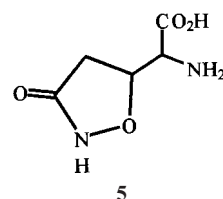


Fig. 5. Chemical structure of tricholomic acid.

Other active substances

(–)-R-4-hydroxy-pyrrolidone(2) **4**, whose structure is closely related to ibotenic acid and muscimol, was found in *Amanita muscaria* (Matsumoto *et al.* 1969a). This chemical frame is common in some micromycetes; they generally exhibit a potent biological activity against bacteria and other fungi (e.g. aureothrycin, equisetin). This additional compound, gives information on the biogenesis of ibotenic acid, muscimol, and muscazone. All probably originate from the same precursor, β -hydroxyglutamic acid; ring closures and decarboxylation then determine the structures of these products (Fig. 4).

Tricholomic acid **5**, a closely related dihydro derivative of ibotenic acid, has been identified in *Tricholoma muscarium* (Takemoto & Nakajima 1964b), and its structure was confirmed by synthesis (Iwasaki *et al.* 1965) (Fig. 5).

Stizolobic **6** and stizolobinic acids **7**, known in *Stizolobium hasjoo* (Hattori & Komamine 1959, Senoh, Imamoto & Maeno 1964), have been found in *A. pantherina*, but not *A. muscaria*, low levels have been detected in *A. gemmata* (Chilton, Hsu & Zdybak 1974). Stizolobic acid, and to a lesser extent stizolobinic acid, exhibit an excitatory action in an isolated rat spinal cord (Ishida & Shinozaki 1988). Biosynthetic studies show that both these amino acids originate from

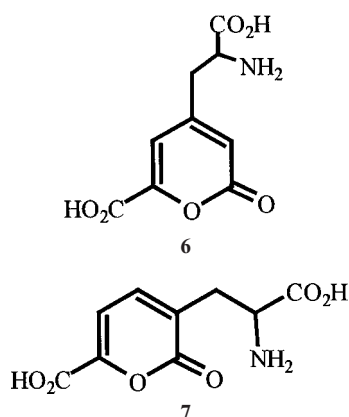


Fig. 6. Chemical structure of stizolobic and stizolobinic acids.

3,4-dihydroxyphenylalanine (DOPA) (Saito, Komamine & Senoh 1975, 1976, Saito, Komamine & Hatanaka 1978), and the enzymes implicated have been isolated and purified (Saito & Komamine 1978) (Fig. 6).

Muscarine has long been considered as the active principle in *A. muscaria* (Schmiedeberg & Koppe 1869, Kögl *et al.* 1957, Cox *et al.* 1958), due to a presumed action on the central nervous system. This was after discredited. Nevertheless, some publications still wrongly assert muscarine's involvement in the action, although several analyses have demonstrated that this agent, which occurs in minute amount in the *Amanita*'s of concern, is not responsible for the poisoning syndrome (Eugster & Müller 1959, Catalfomo & Eugster 1970b). The occurrence of only small amounts of muscarine **8**, i.e. tetrahydro-4-hydroxy-*N,N,N*-5-tetramethyl-2-furanmethanaminium, (0.0002–0.0003%, f.w.; chloride: stout prisms, mp 180–181 ° (Kögl *et al.* 1957, Cox *et al.* 1958), readily soluble in water) have been established in *A. muscaria* (Eugster & Schleusener 1969, Catalfomo & Eugster 1970a). They have to be considered minor components in comparison to some *Inocybe* spp. (to 0.43% in *I. substricta*) and *Clitocybe* spp. (to 0.15% in *C. dealbata*). The two *Amanita* species would not be able to induce any muscarinic syndrome without excessive consumption.

Equal quantities of the muscarine, showing the (+)-(2*S*,3*R*,5*S*) conformation, and (+)-(2*S*,3*S*,5*S*) *epi*-muscarine **9** have been detected in *A. pantherina* (Stadelmann, Müller & Eugster 1976a), as well as (–)-(2*S*,3*R*,5*R*) *allo*-muscarine **10** in *A. muscaria*. Although its occurrence was not demonstrated unambiguously, (+)-(2*S*,3*S*,5*R*) *epiallo*-muscarine **11** was not present in detectable amounts (Eugster & Schleusener 1969, Schleusener & Eugster 1970). Muscarine **8**, *epi*-muscarine **9** and *allo*-muscarine **10** have been detected in the mycelium of *A. muscaria* culture (Stadelmann, Müller & Eugster 1976b). Chemical syntheses of these compounds have been performed: *epiallo*-muscarine **11** (Corrodi, Hardegger & Kögl 1957), DL-muscarine and DL-*allo*-muscarine (Matsumoto, Ichihara & Ito 1969b) (Fig. 7).

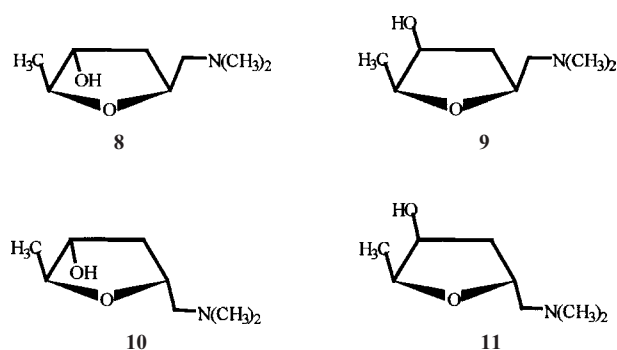


Fig. 7. Chemical structure of muscarines.

Two additional active components reported in *A. pantherina* are (2*R*), (1*R*)-2-amino-3-(1,2-dicarboxyethylthio) propanoic acid **12** and (2*R*), (1*S*)-2-amino-3-(1,2-dicarboxyethylthio) propanoic acid **13** (Fushiya *et al.* 1993). These are antagonists of *N*-methyl-D-aspartic acid (NMDA) receptors KI-II-A and KI-II-B respectively. Actions of these substances could be an alternative explanation of some additional effects observed during *Amanita* poisoning (Fig. 8).

Other active constituents detected in *A. muscaria* include choline, acetylcholine, betain, muscaridin, a quaternary trimethylammonium salt of 6-amino-2,3-dihydroxy-hexan, hercynin, (–)-*R*-hydroxy-4 pyrrolidone, uracile, hypoxanthin, xanthin, adenosin, a carbolic derivative, and β-D-*n*-butylglycopyranoside (Kögl, Salemink & Schuller 1960). Minor amounts of tropan alkaloids, atropine, hyoscyamine, scopolamine, and bufotenine have also been reported (Manikowski & Niezgodski 1962, Depovere & Moens 1984), although their occurrence is rejected by others (Brady & Tyler 1959, Subbaratnam & Cook 1963, Tyler & Gröger 1964, Eugster 1969). The occurrence of muscarine, muscaridine and choline was confirmed, when neither 1-hyoscyamine nor atropine were detected in Dutch specimens (Saleminck *et al.* 1963). The possible occurrence in *A. muscaria* of amatoxins and phallotoxins, the typical toxins of *A. phalloides*, were at first discounted (Catalfomo & Tyler 1961). However, highly sensitive detection methods, radio immunoassays, demonstrated traces of amatoxins (Faulstich & Cochet-Meilhac 1976), and phallotoxins are also suspected (Larcen 1979). Another possibly active compound, bufotenine (5-hydroxy-*N,N*-dimethyltryptamine), has been proposed (Waser 1967) but the occurrence of indolic hallucinogenic substances such as psilocybine frequently speculated, has not been confirmed.

Pigments

The bright colour of *Amanita muscaria* has prompted investigations in several research groups; nowadays most of the structural features of the pigments have been elucidated (Musso 1982). Muscarufin **14** is the principal component; it is probably a putative derivative of a terphenylquinone (Kögl & Erxleben 1930); but

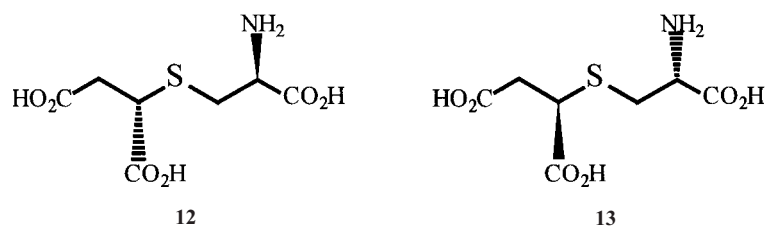


Fig. 8. Chemical structure of (2*R*),(1*R*)-2-amino-3-(1,2-dicarboxyethylthio) propanoic acid and (2*R*),(1*S*)-2-amino-3-(1,2-dicarboxyethylthio) propanoic acid.

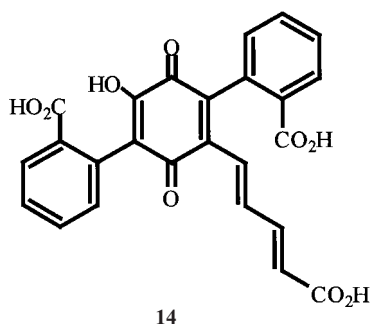


Fig. 9. Putative chemical structure of muscarufin.

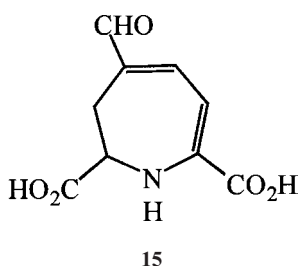


Fig. 10. Chemical structure of muscaflavin.

efforts by different groups to reproduce these evidently premature results failed (Musso 1979) (Fig. 9).

Musso also proposed a quite novel structure, muscaflavin **15**, for a yellow pigment, a result corroborated by a biosynthetic pathway that includes prior divergent oxidative cleavages of L-DOPA, further cyclization steps generating muscaflavin together with betalamic acid, stizolobic acid, and stizolobinic acid (Mabry 1967, Saito, Komamine & Senoh 1975, 1976, Saito, Komamine & Hatanaka 1978). The structure has been confirmed by synthesis (Barth, Kobayashi & Musso 1979, Burger *et al.* 1981). Muscaflavin has also been detected in red-fruited *Hygrocybe* species (von Ardenne *et al.* 1974, Steglich & Preuss 1975) (Fig. 10).

Different compounds, the muscaaurins I–VII **16**, have been proved to be the pigments responsible for the characteristic red-orange colour of the caps of several of other *Amanita* species such as *A. caesarea* (Döpp & Musso 1973). An elegant chromatographic procedure enables the separation of these pigments (Döpp & Musso 1974). Interestingly, ibotenic and stizolobic acids are essentially elements added to the whole structure of muscaaurins (Döpp *et al.* 1982, Depovere & Moens

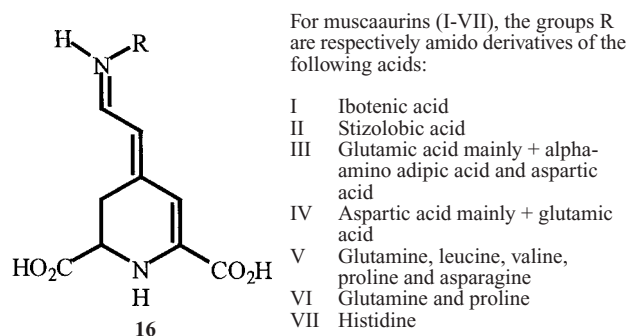


Fig. 11. Chemical structure of muscaaurins.

1984). The muscaaurins are betalaines and also originate from the transformation of DOPA (Fisher & Dreiding 1972). Muscapurpurin (purple) and muscarubin (red-brown), whose structures are closely related to the muscaaurins, have been isolated from *Amanita muscaria* (Döpp & Musso 1973, Musso 1979) (Fig. 11).

Miscellaneous

Dioline 1,3 **17**, a-diester of glycerol and oleic acid; has been considered the putative fly attractant action of *Amanita muscaria* (Muto & Sugawara 1970) (Fig. 12).

Mushrooms are known for differential metal bioaccumulation. *A. muscaria* may concentrate vanadium to 200 ppm (30 times those reported in living organisms). Isolation, structure determination and synthesis of the pale blue vanadium complex, amavadin **18** (Kneifel & Bayer 1986), was confirmed by crystallography (Berry *et al.* 1999). Similarly, unusually high levels of selenium (to 17.8 ppm; Watkinson 1964) and heavy metals have been reported: cadmium 13.9, cobalt 2.6, chromium 1.7, lead 33.3, mercury 61.3, and nickel 7.5 ppm d.w. (Michelot *et al.* 1998, Siobud *et al.* 1999) (Fig. 13).

A lectin (APL) has been isolated from *A. pantherina* through Fast Protein Liquid Chromatography (FPLC), the molecular mass was estimated at 43 kDa and consists of two identical subunits (Zhuang *et al.* 1996). A β -(1 $\rightarrow$ 6) branched (1 $\rightarrow$ 3)-beta-D-glucan (AM-ASN) was isolated from *A. muscaria*. AM-ASN exhibited antitumour activity against Sarcoma 180 in mice (Kiho *et al.* 1992). Phallolysin, a typical haemolysin initially found in *A. phalloides* and able to agglutinate red blood cells has also been detected in *A. muscaria* (Seeger, Kraus & Wiedmann 1973).

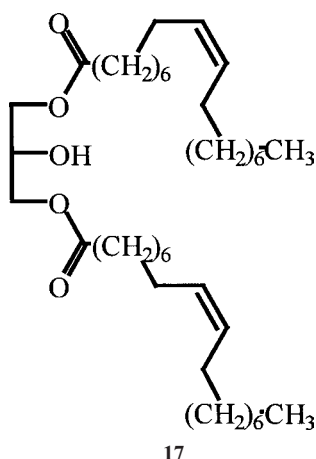


Fig. 12. Chemical structure of dioleine-1,3, diester of glycerol.

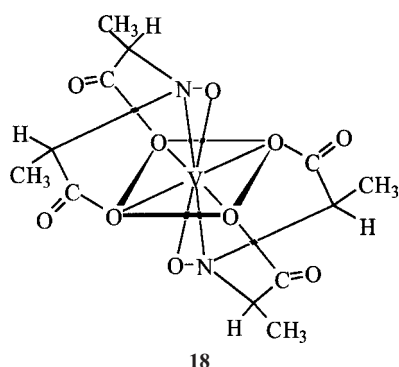


Fig. 13. Chemical structure of amavadin.

Toxin detection, content, and occurrence

Several techniques provided information on the active components in fungi; they are useful for both forensic science and chemotaxonomy (Dorizzi *et al.* 1992).

Due to efforts to obtain pure muscarine, the estimation of the total muscarinic activity and potency of mushrooms was formerly performed by bioassays on rats (salivation and lacrymation; Malone *et al.* 1961, 1962). Detection and quantitative analysis of muscarine in *Inocybe* species has been undertaken by paper chromatography (eluent: *n*-butanol/methanol/water 10/3/2 v/v, staining Thies & Reuther's 1954 reagent; Brown *et al.* 1962). The isomer ratios were detected in the same genus by gas chromatography of the norbases (Catalfomo & Eugster 1970b). Muscarine has been identified in the red caps of Mexican fly agarics by cellulose column chromatography and identification as muscarinetetrachloroaurate (Eugster & Müller 1959).

Isoxazole derivatives have been explored in various *Amanita* species by two-dimensional paper chromatography (eluents: 1, *n*-butanol/acetic acid/water 1/1/1; 2, butanol/pyridine/water 1/1/1 v/v, staining: ninhydrin; Benedict, Tyler & Brady 1966). Ibotenic acid and muscimol were detected in *A. muscaria* and *A. pantherina*, and in intergrades of *A. pantherina* and *A. gemmata*, but not in *A. strobiliformis* by this method. Some

probable isoxazole derivatives were detected in *A. solitaria*. Subsequently, Benedict *et al.* (1966) suggested that the latter substances are chemotaxonomical markers for the differentiation of species and even subspecies. High performance thin layer chromatography (HPTLC) on silica gel allows the detection of muscarine (eluent: *n*-butanol/ethanol 95%/acetic acid/water 80/20/10/30, chromogenic reagent: modified Dragendorff) and muscimol (eluent 2-butanol/ethanol 95% acetic acid/water 75/25/5/25, a chromogenic reagent often used for amino acids). HPTLC permits an evaluation of muscimol equivalents since the experimental procedure, extraction with formic acid, facilitates the decarboxilation of ibotenic acid. The levels of muscimol detected in the samples were of 0.19% d.w. (*A. muscaria*), and 0.3% d.w. (*A. pantherina*). Stijve (1981) made clear the amounts of muscarine were very low (0.009% for *A. muscaria* and lower than 0.0005% for *A. pantherina*). On the other hand, some *Inocybe* and *Clitocybe* species, responsible for the typical muscarine syndrome contain this substance in the range of 0.1–0.3% d.w.

A very sensitive analysis of muscimol and ibotenic acid can detect down to 30 ng of the compound. This technique makes use of an automated amino acid analyser equipped with a microbore column. The highest content of toxins is reported to be in the yellow flesh beneath the cuticle, ibotenic acid: 548 nmol g⁻¹ f.w. and muscimol: 366 nmol g⁻¹ f.w. (Gore & Jordan 1982).

HPLC has been developed for *A. muscaria* content analysis (Lund 1979). Ibotenic acid was assayed on two columns connected in series: (1) Ibotenic acid, LiChrosorb-NH₂ 10 µ column (Merck, Darmstadt, Germany) and a Nucleosil 5CN (Macherey Nagel, Düren Germany) with 0.05 M sodium acetate buffer pH 4 as eluent; (2) muscimol on LiChrosorb-NH₂ 10 µ column, 0.05 M with sodium acetate buffer pH 4/methanol 1/9 v/v as eluent. The amount of ibotenic acid was estimated at 100 ppm f.w. and that of muscimol <3 ppm. Mushrooms extracts treated with acetic acid had muscimol at 40 ppm, demonstrating that ibotenic acid is the major component in the fungus, while muscimol is the real psychotropic substance resulting from decarboxilation occurring during the preparation of the meal and the gastric digestion. Therefore, the two substances must be considered as a functional entity, so-called 'pilzatropine'. An ion-interaction HPLC method permitted the simultaneous detection of muscimol and ibotenic acid. The stationary phase was a reverse C₁₈ matrix, and the mobile phase aqueous octylammonium *o*-phosphate 5 mm. The amounts of both compounds were higher in caps than in stems; the mean values for ibotenic acid and muscimol respectively were (f.w.): 990 mg kg⁻¹ (caps), 230 mg kg⁻¹ (stems), and 380 mg kg⁻¹ (caps), and 80 mg kg⁻¹ (stems). These substances were not detected in *A. mappa* (Gennaro *et al.* 1997). A fresh average size fruit body of *A. muscaria* (60–70 g) contains up to 70 mg of

ibotenic acid (Catalfomo & Eugster 1970a, Ott, Preston & Chilton 1975). Reverse phase HPLC (column: Shim-Pack P-NH₂/S 2054 allows the detection of ibotenic acid (limit: 18 ppm) and muscimol (limit: 10 ppm) (Komiyama *et al.* 1985).

Muscimol as its trimethylsilyl (Repke, Leslie & Kish 1978) or trifluoroacetyl derivatives (Michelot, Brouard & Labia 1993) has been detected by gas chromatography coupled with mass spectrometry (GC-MS).

MODE OF ACTION OF THE TOXINS

The 'insecticidal' effect

A large number of publications traditionally refer to the insecticidal properties of *Amanita muscaria*. Early investigations simultaneously described such an unusual property for ibotenic acid, 'agarin', with qualitative experiments (*Amanita* Factor B; Takemoto & Nakajima 1964a, Takemoto, Nakajima & Sakuma 1964, Takemoto, Yokobe & Nakajima 1964, Bowden, Drysdale & Moge 1965). Moreover a tripeptide, 'pantherine', with such a biological ability has been found in the fungus (Onda, Fukushima & Akagawa 1964). However, quantitative experiments of Catalfomo & Eugster (1970a), who named the substance 'prä-muscimol', showed that diptera were weakly sensitive to any toxic action by contact or ingestion. These results were confirmed by Matile (2000). A putative use of *A. muscaria* components for house fly (*Musca domestica*) control has been discussed with regard to the increasing importance of resistance (Pettsch 1960). Such a reputed characteristic must be rationalized because of the names of the mushroom in different countries and arising comes from oral tradition, tales, paintings, and folklore.

In vivo action of ibotenic related toxins

Besides experimental data on animals, there are a few veterinary reports of accidental cat poisonings, with early symptoms 15–30 min after ingestion (Ridgway 1978). A state of excitement, which lasts up to 4 h, quickly follows a brief period of drowsiness. The animal then passes into a deep sleep, and recovery is usually within 24 h.

Considering the diagnosis, biochemical changes develop 30 min after peritoneal injection of aqueous extracts of *Amanita muscaria*, *A. pantherina* and *A. rubrovolvata* into male rats. These are decreases of acetylcholine esterase activity, liver glycogen, blood urea nitrogen, together with increase of blood glucose level; serum transaminase activities were not affected. The values returned to normal within 6 h (Yamahura *et al.* 1983). The latter data demonstrate that the poisoning is not serious and that vital organs like liver and kidneys are not affected. Vegetative functions are hardly influenced by the toxins. A bioassay was designed to monitor the efficiency of extraction and purification procedures, the prolongation of sleeping time

in mice after administration of a short-acting narcotic (Müller & Eugster 1965). Further studies show that a muscimol active dose given orally is 7.5–10 mg, and the LD₅₀ (i.p.) is 2.5 mg kg⁻¹ for mice and 3.5 mg kg⁻¹ for rats (von Theobald *et al.* 1968). After intraperitoneal injection and oral administration of muscimol (4–8 mg kg⁻¹) and ibotenic acid (16 mg kg⁻¹), the diameter of mouse pupils is broadened. Both drugs induce a marked anorexogenic action on mice (2–3 mg kg⁻¹ oral) with sedation, hypnosis, muscle twitching and catalepsy (Waser 1967, Matsui & Kamioka 1979). The drug produces electroencephalogram (EEG) alterations that are different from those provoked by hallucinogenic substances such as LSD or mescaline (Worms, Depoortere & Lloyd 1979). The EEG patterns resemble those induced by anticholinergic drugs such as atropine and Ditrane (Scotti de Carolis, Lipparini & Longo 1969). The effects are slightly affected by physostigmine (eserine) (Johnston 1971, Saji & Reiss 1987). Bilateral injections of ibotenic acid into the nucleus basalis of rats cause fewer problems than kainic acid (Pepeu & Casamenti 1991).

Most probably, after ingestion, the low pH gastric fluid hydrolyses ibotenic acid into muscimol; afterwards, the active component proceeds to the brain or is eliminated *via* the systemic circulation. The remnant of the active component in the urine accounts for the tradition of drinking the urine of the shaman or of deers who consumes the mushrooms in some Siberian tribes in order to get a 'second-hand' stimulus (Wasson & Wasson 1957). Both ibotenic acid and muscimol are found in human urine 1 h after consumptions; this observation was confirmed experimentally on mice (Ott, Preston & Chilton 1975).

Thus, *a priori*, an acidic decarboxylation step in the gastrointestinal tract would produce muscimol that afterwards enters the central nervous system *via* the systemic circulation (Brehm, Hjedts & Krogsgaard-Larsen 1972). Ibotenic acid has been shown to be the key component of *A. muscaria* and *A. pantherina* (Lund 1979). No direct toxic action on typical target systems implicated in mushroom poisonings (i.e. liver, kidneys, essential metabolism) has been reported (Michelot & Tebbett 1990, Michelot & Toth 1991, Michelot 1992). Nevertheless, repetitive injections of ibotenic acid induce a transient change in GABA receptor sensitivity (Taira, Uusi-Oukari & Korpi 1993) and might produce lesions in the basal forebrain as it destroys a small percentage of cholinergic cells (Harrington & Wenk 1992); the consequence *in vivo* would be a significant and prolonged learning and memory deficit (Connor, Langlais & Thal 1991). In the case of young mammals, muscimol also disturbs the development of the hypothalamic noradrenaline system (Taira & Smith 1993).

Biochemical mode of action of ibotenic related toxins

Ibotenic acid, and particularly muscimol, have to be regarded as the substances responsible for the

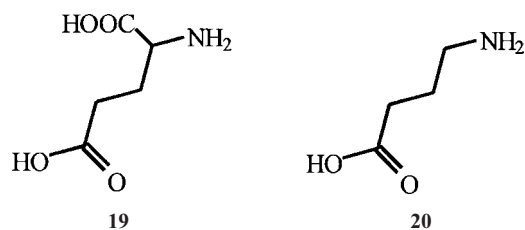


Fig. 14. Chemical structure of glutamic acid and GABA.

psychotropic action of *Amanita muscaria*. The effects of both substances are similar but not identical to the effect of the fungus *in toto*. Ibotenic acid and muscimol are members of a distinctive class of alkaloids, the excitatory amino acids. This group includes kainic acid, domoic acid, tricholomic acid, quisqualic acid, yunaine, and petalonine; all naturally occurring amino acids (Takemoto 1978, McGeer & McGeer 1981, 1983).

Ibotenic acid and muscimol are respectively conformationally restricted derivatives of glutamic acid **19** and gamma-aminobutyric acid (GABA) **20**. Consequently, they act like neurotransmitters involved in the control of neuronal activity in the mammalian central nervous system, operating on spinal neurones (Curtis, Lodge & McLennan 1979). Ibotenic acid is known to act on glutamic acid receptors, and muscimol acts on GABA receptors in the snail *Helix aspersa* (Walker, Woodruff & Kerkut 1971). The same effect is observed in cats; ibotenic acid and excitant action, muscimol and depressant action (Johnston *et al.* 1968). Nevertheless, unlike glutamic acid and GABA, ibotenic acid and muscimol cross the blood–brain barrier (Olpe & Koella 1978), most probably by active transport, counterfeiting endogenous neurotransmitters and causing brain disorders (Fig. 14).

Glutamic acid is a major excitatory neurotransmitter in mammalian central nervous systems and its receptors are implicated in neurological disorders such as epilepsy and Huntington's disease. Inhibitory glutamate receptors (IGluRs) constitute a class of ion channel proteins equivalent to glycine and GABA receptors. Ibotenic acid acts on IgluRs (Cleland 1996). Considering the minimum energy of the mono-anion, ibotenic acid displays a rigid structural analogue of L-glutamate. Hence it presents the suitable shape of glutamate agonists confirmed by molecular orbital theory (Borthwick & Steward 1976). The central inhibitor receptor necessitates an effector displaying two highly charged sites of opposite signs separated by approximately 6 Å (Kier & Truitt 1970); this conformation was confirmed by X-ray analysis (Brehm, Hjeds & Krosggaard-Larsen 1972). AMPA, a synthetic analogue of ibotenic acid, displays the same typical moiety and was found to be more effective and specific glutamate agonist than the parent compound (Watkins, Krosggaard-Larsen & Honoré 1990). Intraperitoneal injections of muscimol and ibotenic acid produce an increase of serotonin and dopamine in the brain of mice and rats (König-Bersin *et al.* 1970, Gundlach & Beart 1980).

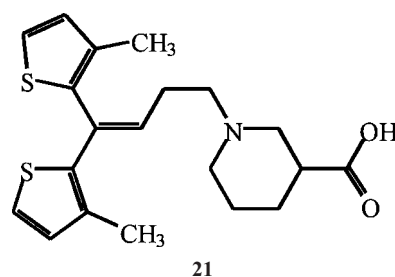


Fig. 15. Chemical structure of tiagabine.

These variations might result from reduced turnover of neurotransmitters, therefore elevations could contribute to psychotonic actions and even accentuate some of them (e.g. anorexia, central pupil dilatation).

There is evidence that damaged function of GABA-mediated inhibitory synapses is implied in experimental and clinical seizure disorders. Decreased concentration, and then activity of GABA, may have a role in human epilepsy. Muscimol is a potent conformational analogue and biologically active bioisostere (Krosggaard *et al.* 2000) (Fig. 15).

This toxin tightly binds with the gamma-aminobutyrate receptor (de Feudis 1980). Moreover, it is also an inhibitor of neuronal and glial GABA uptake and a substrate for the GABA-metabolizing enzyme: GABA transaminase. Subsequently, some research teams tried to improve the potency of drugs. Lipophilicity is of considerable interest for the prediction of the transport, adsorption and distribution properties of molecules, and so is an important factor in drug design. More lipophilic bioisosteres of muscimol and GABA have been synthesized, such as Tiagabine **21**. This drug was marketed as a therapeutic agent for the treatment of epilepsy, Gabatril® (Krosggaard-Larsen 1977, Krosggaard-Larsen *et al.* 1994, Krosggaard-Larsen, Frölund & Frydenvang 2000) (Figs 16, 17).

The above biological and pharmacological activities account for the psychotropic effects of *A. muscaria*.

ETHNOMYCOLOGY

Distinctive features of Amanita muscaria

Among all mushrooms species, the fly agaric has long been a source of interest and attractiveness; it embodies the concept of the 'mushroom' and is probably the most illustrated one. Despite an evil reputation, this mushroom, unique in its striking appearance, is the most widely used pictogram for wild mushrooms. Its uses include illustrations of tales for children, and strip cartoons. Christmas tree decorations, pastries, and even tattoos (Davis & Williams 1999) as well as the artefacts (Taylor-Hawsworth 2001) are responsible for this renown. Because of its vivid colour, white spots, sturdy habit and its psychoactive properties, the fly agaric still exerts great fascination. Shamans, sorcerers, and witches made use of it, all classes of officiating 'priests' in different ethnic groups which are

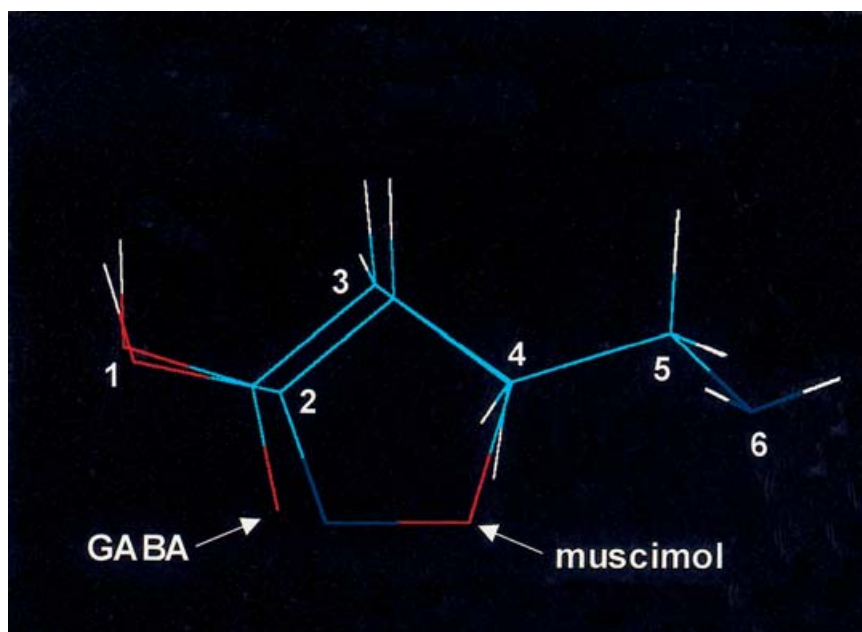


Fig. 16. Chemical conformations ‘3D sticks’ of superimposed molecules of GABA and muscimol. The two structures exhibit a tight-fitting alignment of similar atoms (backbone oxygen 1→nitrogen 6). The conformational flexibility of GABA and the stereochemistry of the bioisostere analogue – muscimol – allow similar affinities for the GABA transporters.

geographically and culturally distant. The ‘priests’ try to escape the human condition and use the mushroom in ‘religious’ rituals. Beside the chroniclers of the 18th and 19th century in northern Europe and Asia (Heim 1964), Wasson (1959) forcefully argued that this mushroom represents the ‘soma’ of some ancient Aryan tribes. This concept designates a ‘plant’ that produces a juice when squeezed, that is thereafter filtered and handled as a divine inebriating agent. He stated that several parts of the Rig Veda conveyed that ancient peoples extensively consumed the mushroom for its

psychoactive properties. Besides various symbols that might correspond to *A. muscaria* and originate from northern and southern Asian traditions, some may also be discerned in Buddhist myths. They appear to be echoed in Germanic tradition, possibly in some characteristics related to the god Odin (Hajicek-Dobberstein 1995). A few publications argue that Christianity originates from a cult of *A. muscaria*, Jesus was supposedly being invested with the energy of the mushroom (Allegro 1970, 1971), but other religious and secular biblical scholars discredit this (King 1970). Nowadays,

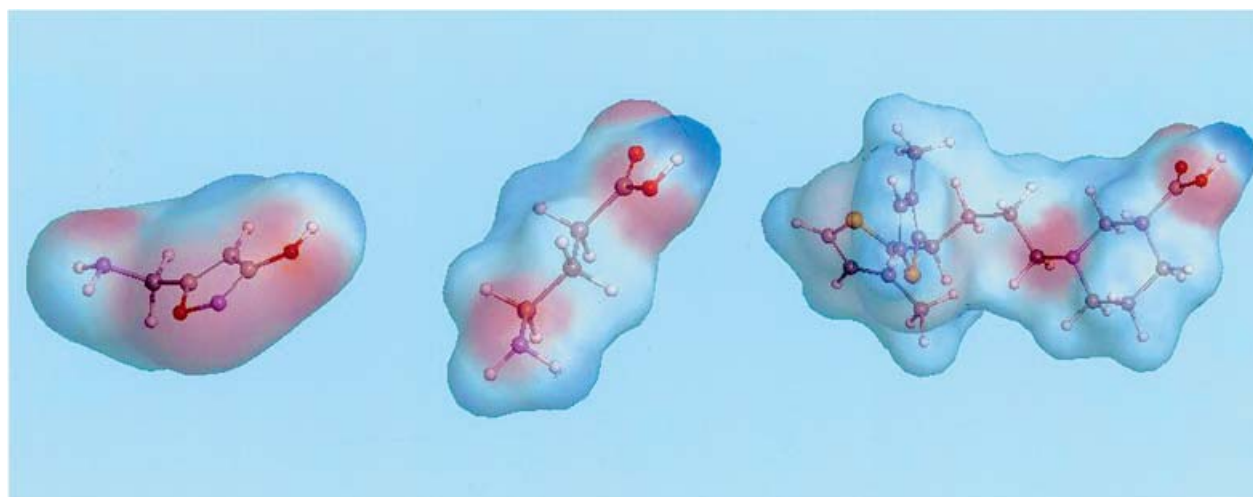


Fig. 17. Molecular modelling of muscimol, GABA and tiagabine displaying solvation surfaces. Minimization was achieved by the software Hyperchem 4[®] software (Hypercube) with force field ‘MM2’ and molecular dynamics at the lowest energy. Visualization of 3D structures was completed by the software Weblabviewer[®] (MSI). Blue and red colours represent negative and positive charges, while white represents zones with high lipophilicity. Muscimol possesses a similar backbone to GABA with corresponding charge scattering. Although tiagabine contains a pyrrolidine ring (vs a linear chain for GABA), the models emphasize similar conformations and distributions of electrostatic charges (at the right side). The existence of two thiophenyl rings (at the left side) yields the proper lipophilicity that helps tiagabine to cross the blood–brain barrier.

reliable sources report that Ostyak and Vogul tribes in western Siberia, and Kamchadal, Koryak, Chukchi tribes in eastern Siberia, still use *A. muscaria* for shamanistic rites. However, in contrast to hallucinogenic mushrooms, mainly species of *Psilocybe* used in Mesoamerican culture (Heim & Wasson 1958, Guzman *et al.* 2000), the fly agaric is not a true object of religious and ritual veneration (Nyberg 1992).

Two attitudes coexist in world cultures towards mushrooms consumption: mycophilic and mycophobic. They are present traditionally among the word cultures. Some groups (e.g. German, Celtic, British) have a dislike for mushrooms, while others (e.g. Slavic, Lithuanian, French, Italian, Spanish, and other Mediterranean ones) are, to a certain extent, fond of them (Levi-Strauss 1970, Festi 1985). The mycophobic attitude possibly corresponds to remnants of a primitive worship/fear of mushrooms connected with 'folk wisdom'. This feeling is conveyed in language and etymology, possibly starting from Neolithic and Palaeolithic times. Unconsciously, some people make a clear-cut distinction between 'safe' mushrooms and suspicious mushrooms for instance mushrooms *vs* toadstools in English, and setas, hongos *vs* hongos venenosos in Spanish. The different attitudes are documented by Lowy (1974) who travelled widely in Latin America and various steps in the mycophobia/mycophily scale (Heim & Wasson 1958). Moreover, educated people are careful about mushrooms, when those of less developed cultures tend to consume them more readily.

The name of the mushroom

The designation of the species as 'fly agaric', 'bug agaric', is surprisingly equivalent all over the world: 'amanite tue-mouche' (French), 'Fliegenpilz' (German), 'muchomor' (Russian), 'moscario' (Italian), 'hongo mosquero', 'hongo matamoscas' (Spanish). The name of *Amanita strobiliformis* in Japanese is 'haetorimodashi', fly-killer). At the beginning of the last century, Atkinson (1901) mentioned the practice concerning a preparation of the mushroom to kill flies. The presumed insecticidal effect against flies should, however, be discounted since experiments show that a percentage of flies exposed to the juice of the mushroom did not die, but were rather subjected to transient behaviour disorder (i.e. became intoxicated). However, a few experiments do show that some species of *Amanita* inhibit the growth of *Drosophila melanogaster* larvae fed with powdered fruit bodies (Besl, Krump & Schefcsik 1987).

The 'fly' name may be explained in another way, since it bears the same semantic sound in several languages. Going back to ancient times in many countries, 'fly' has been identified with madness or supernatural possession. In the Middle Ages, it was believed that flies or other insects could penetrate the head of a person and cause mental illness. This is evidenced in Hieronymus

Bosch's painting 1510 'Paradise and Hell': on the left panel, one can see very well high above in the sky the rebellious angels, who flew from heaven as a crowd of hideous insects. The Prado Museum in Madrid exhibits another painting by the same artist 'The garden of earthly delight' 1504. *A. muscaria* can be distinguished on the left-hand panel of the work (Heim 1968). In Europe, some other terms naming the mushroom are reminiscent of madness, for example 'Narrenschwamm' (German), 'oriol fol' (Catalan), 'mjioulo folo' (Toulouse dialect), 'coucourlo foulo' (langue d'oc), and 'ovolo matto' (northern Italian) (Festi 1985). Conversely, the fly-possession link could be rather beneficial to shamans. The Bible is no exception; 'Evil One', Belzebuth, means 'Lord of the flies' and is synonymous with a false God adored by drunkards. It is likely that *A. muscaria* was named 'mushroom of the flies' because it is able to induce divine or evil states of possession similar to those that flies were supposed to produce in human brains. In Mexico, a Quiche idiom that describes hazardous, frightful potentialities is 'itzel cox', meaning the diabolical mushroom (Lowy 1974). The original meaning was evidently forgotten or misinterpreted over time, giving way to the so-called insecticidal activity.

With respect to rituals and beliefs, the habitat of the fly agaric is pertinent. The tree has long been considered the 'Tree of Life', identified with the 'Weed of Immortality', and later the 'Tree of Knowledge' and with the 'Forbidden Fruit' in the Genesis (Levi-Strauss 1970, Festi & Bianchi 1992). Thus, it appears to be at the origins of religious practices (Wasson 1959). Its roots were said to 'swallow the pool of water of life' and nourish the 'sacred mushroom', 'his mind is the mind of a woman who offers her milk to anybody that comes towards her' (Wasson & Wasson 1957, Festi 1985). *A. muscaria* is often mycorrhizally associated with *Betula*, and also *Abies*, *Cistus*, *Picea*, *Pinus*, and *Quercus* species (Pegler 1983, Castro 1998, Contu 2000, Curreli 2000).

The appearance of the mushroom is unique, colourful, red and covered by whitish patches. It would be possible to consider this aposematic as it is seen in ladybirds, dendrobate frogs, etc. The conspicuous markings make it easily recognizable and warn would-be predators that it contains toxins.

Paintings, frescos, and archaeological data

Historical documents, such as wall paintings, wood-carvings, and sculptures suggest that the psychotropic properties of some mushrooms have been known from ancient times and that consumption was for religious, social and therapeutic purposes, and occurred in all continents (Heim 1964, Festi 1985). Representations of *Amanita* mushrooms, most probably *A. muscaria*, have been reported in polychromatic rock paintings in the Sahara (Samorini 1992); such works date from the Palaeolithic 9000–7000 BC (Heim 1964, Festi 1985).

The American surgeon general Puharich (1959) was particularly interested in extrasensory perception after World War II and searched for psychotropic substances able to induce latent abilities among 'sensitive' subjects. He investigated the mycology, biology, physiology, ethnology, etymology, and comparative linguistics of *A. muscaria* and its psychotropic properties. Further, he attempted to prove there were cults in ancient Egypt equivalent to those of the Indo-European transmutations, and present today in eastern Siberia. Puharich also noted the comparison with cults in Central America related to hallucinogenic mushrooms. His conclusions confirm those of Samorini (1992) relating to other parts of north Africa. Among the various pieces of evidence he produced is an unambiguous distinction between a mushroom, a sunshade, and a lotus flower on seals illustrating a ritual ceremony in the pre-Snefrou period (before 2200 BC).

CONCLUSION

Amanita muscaria occupies a unique position amongst all mushrooms. Its emblematic aspect merges with the psychotropic effects and the chemical load. As far as toxic effects are concerned, consumption of *A. muscaria* and *A. pantherina* does not induce any critical organ damage. Thus, in the case of such a poisoning, the victims are not considered endangered, but intensive care is recommended. Nevertheless, severe neuron and even brain lesions could be anticipated in cases of recurrent consumption.

A. muscaria has contributed to the progress of chemistry and pharmacology. Noteworthy, is that in most European countries, the possession, sale and consumption of the species are not yet subject to legislation such as in place for the more widely recognized hallucinogenic species (i.e. *Psilocybe*, *Panaeolus* and other species containing psilocybin-related toxins). *A. muscaria* is not yet the object of any drug-traffic, an aspect reflected in the lack of awareness amongst possible consumers or applicants for recreational purposes. Considering the effects so far reported, *A. muscaria* has a low psychotropic action, but still a toxic one.

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Short communication

Fly agaric (*Amanita muscaria*) poisoning, case report and review

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Abstract

Gathering and eating mushrooms and other plants containing psychoactive substances has become increasingly popular among young people experimenting with drugs. Dried fly agaric *Amanita muscaria* fruiting bodies were eaten by five young persons (18–21 years of age) at a party in order to evoke hallucinations. Visual and auditory hallucinations occurred in four of them, whereas a 18-year-old girl lost consciousness. The following morning, she went to the Clinic of Toxicology. Due to the fact that not all the active substances present in the fly agaric have been identified, and some of them have an effect after a period of latency, the patient was admitted for several days of observation during which check-up examinations were performed. After four days without any problems, she was discharged. The poisoning regressed with no organ complications. The remaining persons who had eaten the fly agaric were free from any complaints.

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1. Introduction

Man has been taking drugs for thousands of years. People have always been interested in substances that have an effect on the mood, perception and the cognition of a new reality. The psychotropic properties of *Amanita muscaria* have been known since ancient times. The use of the fly agaric is connected with mysticism. In Poland, there are plants such as *Datura stramonium* and hallucinogenic mushrooms that have become an increasingly frequent cause of poisoning, especially in young persons who experiment with psychoactive substances. In summer and autumn, mushroom poisoning is a frequent cause of hospitalisation in the Clinic of Toxicology (Satora, 2004).

In Poland, the fly agaric cannot be mistaken for any other mushroom. *A. muscaria* is commonly found in birch and coniferous tree forests throughout Poland from July to November. Its fruiting bodies reach up to 200 mm in height, whereas its caps—even up to 200 mm in diameter. Fly agaric poisoning happens accidentally, especially in children; however, it is also a cause of deliberate poisoning. Recently, toxicological centres have increasingly dealt with the practices of getting intoxicated through drinking infusions made from those mushrooms or eating the whole fruiting bodies.

2. Case study

Six large fruiting bodies of the fly agaric were gathered in a city park in Kraków one morning. The persons who gathered them used a key to mushrooms. Then the caps were

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skinned, shredded and dried in an oven. On the same evening, the mushrooms were served at a party. They were eaten with bread and salad by five persons. The participants drank a can of beer each. The first clinical manifestations appeared after about 20 min. Each person (between the ages of 18–21, four males and a female) experienced auditory and visual hallucinations (heightened colour perception). Disturbing reactions occurred in the 18-year-old girl. Initially, she had severe hallucinations, followed by loss of consciousness. Other partygoers induced her to vomit and made her drink liquids. Those who had also eaten the mushrooms exhibited no symptoms. Due to general debility, tiredness and gastric pain, next day she went to the Casualty Department of the Clinic of Toxicology, Medical College, Kraków. The patient was weak; palpation revealed tenderness in the stomach. Bilirubin level was slightly elevated, whereas amino-transferases and prothrombin index were within normal limits. No other abnormalities or metabolic disorders were found in the laboratory investigations of hepatic and renal functions. There was a regular sinus rhythm of 60/min, PQ 113 ms, QT 400 ms in the ECG; the tracing was normal. Urine samples were collected 5 h after mushroom ingestion, analysis for THC, amphetamine, opiates and amanitin was performed. The results were negative. Mycological analysis was made and the species of the poisoning-inducing mushroom was determined. Due to the fact that not all the active substances present in the fly agaric have been identified, and some of them have an effect after a period of latency, the patient was admitted for a several-day observation during which check-up examinations were performed. Since the patient complained of gastric tenderness, abdominal ultrasound examination was carried out. The examination revealed two hyperechoic foci—steatosis—in the right hepatic lobe. On the following day, a psychological evaluation was performed. The patient admitted having experimented with marihuana, hallucinogenic *Psilocybe* mushrooms and drunk beer occasionally. It was the first time she had tasted the fly agaric, but its effect made her refuse to take it any more. The patient was diagnosed with puberty-related emotional problems; she was provided with follow-up psychological care. In the treatment the patient was administered activated charcoal and nifuroxazide. She received also potassium chloride and fed light diet.

3. Discussion

Despite numerous studies of the substances present in the fly agaric, the correlation between the physicochemical properties of the active components and the mode of consumption is still unclear. Ibotenic acid (pantherine, agarine) and muscimol, which are readily soluble in cold water, are the main active substances contained in the fly agaric. The red skin of the cap and the yellow tissue beneath it contain the highest amounts of these substances. Another

substance in the fly agaric is muscazone, which is a lactame isomer of muscimol. The substances have effects on the central nervous system (tiredness, confusion), but they act with a longer latency period. Other substances found in the fly agaric are: choline, acetylcholine, betaine, muscaridine; minor amounts of tropane alkaloids: atropine, hyoscyamine, scopolamine and bufotenine. The possible occurrence in *A. muscaria* of amatoxins and phallotoxins—the typical toxins of *A. phalloides*—is taken into consideration (Michelot and Melendez-Howell, 2003). Ibotenic acid and muscimol have a structure similar to that of the two main neurotransmitters of the central nervous system: glutamic acid and GABA. Ibotenic acid and muscimol act like neurotransmitters involved in the control of neuronal activity of spinal neurones in the central nervous system (Connor et al., 1991; Michelot and Melendez-Howell, 2003). In cats, ibotenic acid is known to act on glutamic acid receptors, whereas muscimol acts on GABA receptors. Ibotenic acid and muscimol cross the blood-brain barrier, most probably by active transport, causing brain disorders by counterfeiting endogenous neurotransmitters (Michelot and Melendez-Howell, 2003). In most cases, one cap is a sufficient amount for psychotropic effects (Benjamin, 1992). Clinical manifestations typical of the poisoning with the above-mentioned *Amanita* species are those resembling alcohol intoxication. *A. muscaria* and *A. pantheria* were used by witch doctors for inducing hallucinations. In spite of the fact that tropane alkaloids are not present, the signs and symptoms of poisoning with the fly agaric are referred to as ‘mycoatropinic’ and they resemble those produced by *Datura stramonium*, *Atropa belladonna* and *Hyoscyamus niger*. They are also referred to as ‘pantherine-muscarine’ poisoning syndrome. The first manifestations appear 30 min to 2 h after the mushroom consumption. First, there is dizziness, difficulty in balance and movement coordination as well as tiredness that sometimes progresses into sleep. Those are followed by psychomotor excitement with euphoria or depression and anxiety, illusions and visual and auditory hallucinations (Davis and Williams, 1999). The phases of tiredness and excitement may alternate several times. There is an increase in body temperature, the skin and mucous membranes are dry, the face is red and the pupils are dilated. Nausea, vomiting and diarrhoea are quite frequent. In severe poisoning, there is tremor or tonic-clonic seizures with loss of consciousness, followed by a coma. In such cases maximum pupillary dilation, loss of corneal, superficial (e.g. abdominal reflex) as well as deep reflexes (e.g. knee reflex) are often observed. In the most severe cases, life-threatening respiratory and circulatory disorders may occur. Sporadically, muscarine-like manifestations may appear: sweating and hypersalivation. The duration of clinical manifestations does not usually exceed several hours. Retrograde amnesia is a frequent result of the poisoning. The severity of the reaction is proportional to the amount of the mushroom ingested. Tests with rats revealed decreases in acetylcholine esterase activity, liver glycogen,

blood urea nitrogen, together with the increase in blood glucose level; serum transaminase activities were not affected. The values returned to normal within 6 h. Biochemical changes develop 30 min after peritoneal injection of aqueous extracts of *A. muscaria* (Yamahura et al., 1983).

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ETHNOMYCOLOGICAL DATA FROM SIBERIA AND NORTH-EAST ASIA ON THE EFFECT OF *AMANITA MUSCARIA*

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Summary

The paper includes literary data on the use of *Amanita muscaria* in Siberia and North-East Asia as well as information collected from ethnographers investigating these areas during the past decades. A survey is given on the cases and rules of *Amanita muscaria* consumption and the ways of its administration. The peoples having the tradition of *Amanita muscaria* consumption were aware of its different psychotropic qualities and were able to use for several purposes. The fungus has been used by them as a psychostimulant having a simultaneous effect on several psychic functions.

Introduction

The biochemical composition and psychotropic effect of *Amanita muscaria* (Fr.) Pers. ex Hook. are not quite clear (Singer, 1978; Flammer, 1985). For their proper elucidation we might also take into consideration the data originating from animist-shamanist cultures as they are different from those obtained with the help of experiments performed on volunteers and those collected on the basis of casual intoxications.

The following abbreviations are used in the paper: kh, the Khanty; i, the Itelmen; K, Kamchatka; k, the Koryak; AM, *Amanita muscaria*; m, the Mansi; fn, the Forest Nenets; n, the Nganasan; s, the Selkup; Ch, Chukotka; ch, the Chukchi.

Materials

Sources

The paper is based on the following data: (1) literary information from groups of people who have lived on the territories with customary AM consumption from the 17th century up to the first third of the 20th century;

most of it has been represented by Wasson (1968) whose references have been used by the author of the present paper; (2) both oral and written messages from ethnographers, folklorists and archaeologists working on these territories at present; (3) information collected by the author from the Khanty of the Great Yugan (a tributary of the Ob River) in 1987 and from an Estonian who worked as a reindeer herdsman with the Chukchi at the beginning of the 1970s.

Regions of AM consumption and nations involved

Different animist-shamanist cultures of the Asian part of the U.S.S.R. have had different relations to AM. In some cultures AM was regarded as a special object, a supernatural being whose action or influence could be used by man in his particular interests, in others it was not paid any attention whatsoever.

Cultures with customary AM consumption spread in two regions. One of them was West Siberia together with the Taimyr peninsula. AM consumption has been observed among the Khanty (Kamieński Dłuzyk, 1874; Georgi, 1780; Patkanov, 1897; Dunin-Gorkavich, 1904; Karjalainen, 1927; Shatilov, 1931; Yuri Simchenko, 1987; personal communication; Maret Saar, 1987, field work data), the Mansi (Dunin-Gorkavich, 1904; Karjalainen, 1927; Kannisto, 1958), the Forest Nenets (Lehtisalo, 1924), the Selkup (Shatilov, 1927; Donner, 1933), the Nganasan (Yuri Simchenko, 1987), information about the archives of the missionaries (personal communication), and the Ket (Donner, 1933). At the present time there are no data on this phenomenon in this region (kh: Vladislav Kulemzin, Nadezhda Lukina, Edgar Saar, Zoya Sokolova, 1987, all in personal communications; m: Zoya Sokolova, 1987, personal communication; fn: Vladimir Vassilyev, 1987, personal communication; n: Yuri Simchenko, 1987, personal communication). In the regions with the Khanty population where the phenomenon was the most widespread in the past, however, we may assume its episodic existence even now (Maret Saar, 1987, field work data).

The other region included roughly all the continent east of the meridian passing through the mouth of the Kolyma River together with the Kamchatka peninsula. All the nations inhabiting that territory — the Chukchi, the Koryak, the Itelmen, the Eskimoes, the Russians living along the Kolyma for centuries, the Chuvanians*, the Yukagir, the Even — had the similar idea of AM as a supernatural being (Yuri Simchenko, 1987, personal communication). At present the custom of AM consumption is still alive there (Yelena Batyanova, Vladimir Lebedev, Yuri Simchenko, 1987, all in personal communications).

*One of the Yukagir tribes, partly assimilated with the Chukchi and the Koryak.

The relation of AM consumption to the religious position of the consumers and to their sex

In different cultures, sometimes even within one culture, there have been differences depending on the sex and religious standing of the consumers.

With many peoples AM has been used only by persons having a special relation to religion. This is the case with the Forest Nenets (Lehtisalo, 1924); the Selkup (Donner, 1933) and the Ket (Donner, 1933). With most peoples, however, the use of AM was not confined to a certain class and it was also used by ordinary people. Here belong the Chukchi (Dittmar, 1900; Johelson, 1905, 1908; Bogoraz, 1904—1909; and Vladimir Lebedev, Yuri Simchenko, 1987, both in personal communications), the Koryak (Strahlenberg, 1736; Krashennikov, 1755; Steller, 1774; Langsdorf, 1809; Erman, 1833—1848; Maydell, 1893; Dittmar, 1900; Slyunin, 1900; Enderli, 1903; Johelson, 1905, 1908; Bogoraz, 1904—1909; Bergman, 1926; and Yelena Batyanova, Vladimir Lebedev, Yuri Simchenko, 1987, all in personal communications), the Yukagir (Steller, 1774; Johelson, 1905, 1908; and Yuri Simchenko, 1987, personal communication), the Itelmen (Krashennikov, 1755; Steller, 1774; Langsdorf, 1809; Erman, 1833—1848; Dittmar, 1900; Johelson, 1905, 1908; Bogoraz, 1904—1909; and Yuri Simchenko, 1987, personal communication), the Chuvanians (Yuri Simchenko, 1987, personal communication), and the Eskimoes (Yuri Simchenko, 1987, personal communication). The Khanty (Karjalainen, 1927; Shatilov, 1931; and Yuri Simchenko, 1987, personal communication; Maret Saar, 1987, field work data) and the Mansi (Dunin-Gorkavich, 1904; Karjalainen, 1927; Kannisto, 1958) have a few regional differences. Detailed data about the Nganasan are lacking.

In order to prevent erroneous opinions of the significance of AM in magic rites we must state that AM consumption in them was not obligatory. AM was used only by some magicians and not in all rites, while the magicians who could do without AM were considered stronger (Yuri Simchenko, 1987, personal communication).

The sex of a consumer of AM was also important when performing some sacred procedures by an ordinary member of the community.

Usage

AM has been consumed on several different occasions. They can be divided into four groups: sacred and magic activities, recital of epics, work and physical effort, narcotic purposes.

Sacred and magic activities

The use of AM has been reported in connection with the following activities: communication with the souls of the dead (ch: Bogoraz, 1939; K, Ch: Yuri Simchenko, 1987, personal communication), communication with spirits (kh: Patkanov, 1897; Karjalainen, 1927; Shatilov, 1931; m: Karjalainen, 1927;

Chernetsov, 1935; k: Johelson, 1905, 1908; Ch, K: Yuri Simchenko, 1987, personal communication), treatment of diseases (kh: Karjalainen, 1927; Shatilov, 1931), giving a name to a new-born child (ch, k: Vladimir Lebedev, Yuri Simchenko, 1987, both in personal communications), making clear the cause of a dangerous situation and finding ways to overcome it (kh: Karjalainen, 1927; Maret Saar, 1987, field work data; m: Chernetsov, 1935; s: Shatilov, 1927; fn: Lehtisalo, 1924; k: Johelson, 1905, 1908), interpretation of dreams (k: Johelson, 1905, 1908), prediction and fortune-telling (kh: Karjalainen, 1927; ch: Vladimir Lebedev, 1987, personal communication; k: Johelson, 1905, 1908; Kosven, 1962), foreseeing of the future (k: Enderli, 1903; ch: Bogoraz, 1904—1909), seeing of the past (ch: Vladimir Lebedev, 1987, personal communication), visiting of different worlds (k: Enderli, 1903; Johelson, 1905, 1908), flights over the earth (Ch, K: Yuri Simchenko, 1987, personal communication).

According to the world outlook intertwined with religious and magic elements the changes in the world of people are always connected with those in the world of supernatural beings. Man can influence the events of his life working upon these beings. To do this, he must know first whom he has to affect, and then act properly on the basis of right information. In complicated situations and crises it may happen that the information obtained by an ordinary member of the society proves insufficient or he is not able to influence supranormal beings. To enter into the world of the latter and communicate with mighty beings there is the privilege of the persons belonging to the highest sacred rank in the society*. Such people can free one of their souls from the body, send it to the world of supernatural beings, let it act there in the required manner and then bring it back successfully**. The necessary information about people as well as the supranormal world is collected at the initial stage of the procedure, using thereby the aid of spirits coming from the supernatural world (Siikala, 1978). It is characteristic of the procedures that AM is used at the beginning of the whole course of activities. Often the person involved has a sleep after consuming AM and starts influencing the supranormal world only after he has woken up.

Consequently, the process of communicating the world of supernatural beings is diverse and it has a number of stages. There are three kinds of opinions as to which aspect should be stressed with the help of AM at one or another stage.

*At the initial stage of animism any person was allowed to contact the supranormal world. Later this activity became the privilege of special persons (the shamans) whose soul was able to leave the body without doing any harm to the master (Kulemzin, 1984). In the investigated regions there exist world outlooks hardly differing from those characteristic of the initial stages of animism (e.g. the Koryak, the Chukchi); nevertheless, we also came across those of developed ones (e.g. the Khanty, the Mansi).

**"Contacting the spirit world is not only difficult, it is also dangerous. In order to help his band of supporters at critical points in their lives the shaman places his own well-being and even his life — as shamans themselves often say — at stake" (Siikala, 1978).

(1) In the opinion of Siikala, AM is a means of amplifying the rhythmical stimulation of changes in the conscious mind of the shaman*.

(2) Another kind of opinion might be contemplated from the point of view of psychology as "the effect of AM on information processing".

In the world outlook of both regions of AM consumption the fungus is connected with clairvoyance and information obtained from spirits. The typical explanations of the phenomenon are as follows: the fungus brings about a special state in which the consumer "knows everything", e.g., who has stolen something from him, or deceived him, etc. (s: Shatilov, 1927); it helps to understand the language of the spirits (m: Chernetsov, 1935); it has the power, when necessary, to predict the future to the person has eaten it (k: Enderli, 1903; Kosven, 1962, referred according to Novik**); it tells every man, even if he were not a shaman, what aided him when he was sick, or explains a dream to him, or shows him the upper world or the underground world[†]), or foretells what can happen to him (k: Johelson, 1905, 1908); it tells people the cause of the evil, e.g. when a man or an animal has got lost or when somebody is ill (kh: Maret Saar, 1987, field work data); it gives an answer to a question, e.g. how to cure a sick person (fn: Lehtisalo, 1924). According to the Khanty, AM is the only spirit able to predict something (Vladislav Kulemzin, 1987, personal communication).

Information is obtained at the time of hallucinations or dreams. Their course and aim is different in every region but the common trait of all is the appearance of anthropomorphous spirits, the so-called AM-manikins[‡]. They answer the questions asked by the AM consumer (kh: Karjalainen, 1927; Maret Saar, 1987, field work data) and give him information about the problem he is interested in. This is done singing (kh: Karjalainen, 1927) or in the form of a narrative (fn: Lehtisalo, 1924). They take the person by the hand and lead him through the whole world, showing him various things, sometimes taking him even to the world of the dead (ch: Bogoraz, 1904–1909); they show him the future and the past (k: Johelson, 1905, 1908).

*According to Siikala (1978) "the framework of the shaman's technique of ecstasy is similar to the basic psychic mechanism on which hypnosis is founded; this mechanism is undoubtedly common to all goal-oriented trace behaviour. . . The mental process of the trace induction stage can be regarded as a gradual deeping of role-taking directed at the supranormal. . . Mechanical means of stimulating the nervous system are rhythmical music, singing, dancing and drugs. . . The use of hallucinogens and other such intoxicants is not a vital element of trance technique in any part of Siberia. . . The shaman's altered state of consciousness comes into being as generalized reality orientation disappears along with an increase in the intensity of the rhythmical, gradually accelerating sensory stimulus and role-taking directed at the supranormal.

**Dikov, 1984.

[†]According to the Koryak and Chukchi they are, respectively, the future and the past.

[‡]The conception of AM-spirits as beings of the supranormal world, anthropomorphous to a greater or lesser extent, is common in both regions of AM consumption. Probably it goes back to time immemorial (Dikov, 1971; Devlet, 1975). Petroglyphs in Chukotka depicting AM-manikins are supposed to have belonged to the ancestors of the Chukchi, assimilating the proto-Eskimos (Dikov, 1971).

Information is given to the listeners at the time of dreams (kh: Maret Saar, 1987, field work data) or hallucinations (kh: Karjalainen, 1927), sometimes, however, after waking up (kh: Karjalainen, 1927; fn: Lehtisalo, 1924).

The essence and distribution of AM-questioning* on the territory inhabited by the Khanty has been treated by Karjalainen (1927). In his opinion the fungus has been consumed to call forth dreams that would give information and would enable foretelling the future.

Some data are now presented on the Khanty of the Yugan (Maret Saar, 1987, field work data), originating from two old clans whose settlements are situated in the middle reaches of the river, about 200 km from each other. The author's informers were Yakov Petrovich Kuplandeyev (b. 1959) from one family and the Ryskin brothers, Yefim Mikhailovich (b. 1939) and Ivan Mikhailovich (b. 1941) from the other. They all regarded the phenomenon of AM-questioning, in principle, from the same angle. They called it clairvoyance or a fit of second sight.

"AM was used to get inebriation as well as to call forth a fit of second sight for the purpose of learning something definite, for instance: what ails you, where the lost thing, animal or person is and whether the latter are dead or alive. To evoke a fit of second sight, AM was consumed by wise men" (the explanation of Yakov Kuplandeyev).

"This fungus is used to bring about a spell of second sight. For instance, when a child is ill, the parents want to know whether it is a scourge of god or something else. The father of the child eats the AM to evoke such a spell. He falls asleep and in his dream sees manikins as tall as half an average man (the informer stood up and showed to his waist-belt). The manikins ask the man why he has eaten AM. The man explains his wish and the dwarfs give the answer. For some time the man sleeps without dreams and then the manikins appear once more. The man may now ask another question. During one dream the dwarfs may appear several times, each time giving a little more information. To the onlookers it seems as if the man were asleep, only sometimes he speaks in a loud voice (i.e. when he is talking to the manikins). At last the man falls sound asleep and by the time of his waking up the fit of second sight has passed. Only the pileus of the fungus is eaten**. For the purpose of clairvoyance AM is consumed only by men. The following day the consumer may have attacks of sickness and vomiting. If the manikins do not appear, the explanatory vision will not be obtained." Yefim Ryskin who told this had not eaten AM himself. His information was based on his father's experience.

"AM was used when some misfortune happened. People then appealed to a wise man (the Khanty have a special name for him) for help. If there was no wise man in the neighbourhood, people had to try to learn the secret

*The term used by Karjalainen.

**The Khanty of the Yugan eat only the pileus of AM, the part where the stipe is attached, however, is not eaten.

themselves. Only a man was allowed to consume AM. If nothing came of it, one had to look for a wise man after all." (Ivan Ryskin's explanation).

The questioning of the fungus may sometimes fail, the AM-manikins may not turn up (kh: Maret Saar, 1987, field work data) or they may take the man to other countries and places without giving necessary data on the required problem (kh: Karjalainen, 1927).

AM consumption by the shaman is looked upon as the creation of an assistant. It is perceived like the reception of AM spirit into himself (kh: Karjalainen, 1927), whereas the shaman seems to surrender to an external pressure:

"The shaman ('vorozhey') does not eat AM because he wishes to. He is made to eat it by the AM spirit who speaks and acts through him" (kh: Vladislav Kulemzin, 1987, personal communication).

When forwarding the information to the listeners, the shaman feels as if he were only a means in the hands of AM spirit:

"The subject of the old woman's (a woman shaman — M.S.) song was not she herself but 'the spirit of the fly-agaric'" (kh: Karjalainen, 1927).

"When a vorozhey eats AM, then he, the vorozhey, sings. He who sings is not he, the vorozhey, but AM" (kh: Vladislav Kulemzin, 1987, personal communication).

The poetic nature of a Khanty song written down by Heiki Silvet (1988, personal communication) refers to the positive emotional state of the shaman after getting information. The song represents the shaman's appeal to AM as a mediator in communicating with superior beings.

Oh you little gold-stiped fly-agaric, chao-chao-chao,
Such tidings you brought me, chao-chao-chao,
Little patterned-stiped fly-agaric, chao-chao-chao,
Many messages, many words you have, chao-chao-chao,
(An unknown word) is placed on golden grass, chao-chao-chao,
Little duckling, little duck, chao-chao-chao,
One half of you walks on the water, chao-chao-chao,
The other half of you walks on the ground, chao-chao-chao,
Pulling snare, pulling string, chao-chao-chao,
So one must rock, chao-chao-chao,
You are among fly-agarics a little fly-agaric.

(3) According to the Khanty AM is also a means of checking the shaman's fear (of increasing his courage) at the central stage of the procedure, i.e. the stage of communicating with the supranormal world.

"... a bear eats AM during the rutting season in order 'not to fear', and the informer has compared it in this sense with a shaman who eats AM not only for foretelling the future after having dreamed frightening events but also in order to encourage himself before meeting several spirits" (kh: Kulemzin, 1948).

Recital of epics

In animist-shamanist cultures as well as in pre-literary cultures oral

recital of folklore was a widespread feature. Special performers acted for this purpose. Among them AM consumption has been observed to facilitate their activities.

"To inspire himself, a singer (performer of heroic epic songs — M.S.) would eat a few AM before starting to sing...: under their influence he would get simply mad... Then he would sing heroic epics in a ferocious voice all the night long, including even those which seem to have been forgotten long ago; in the morning he would fall on the plank-bed, powerless. The listeners, indifferent to his state, are content to have heard the songs of their fathers in such an emotional presentation" (kh: Patkanov, 1891).

"On the Vasyugan there were special persons, called *pankal-ku*. A *pankal-ku* soaked AM in warm water and drank the extract which had the effect of a soporific and a narcotic. The people present gathered round the sleeping *pankal-ku* and listened to his dream that he was telling in delirium" (kh: Kulemzin, 1976).

"Once I asked a Reindeer Koryak, who was reputed to be an excellent singer, to sing into the phonograph. Several times he attempted, but without success... After eating two fungi, he began to sing in a loud voice, gesticulating with his hands" (k: Johelson, 1905, 1908).

Work and physical exertions

Here belongs the cases where a man increases the resistance of his organism to great physical strain and skill of his movements: pursuit of animals in a canoe (Ch: Bogoraz, 1904—1909), haymaking (K: Erman, 1833—1848), pulling boats with ropes (kh: Yuri Simchenko, 1987, personal communication), carrying heavy loads over long distances (K: Langsdorf, 1809), long walking tours (a Cossack: Krashennnikov, 1755; K: Vladimir Lebedev, 1987, personal communication; Ch: Bogoraz, 1904—1909) or running (Ch, T: Yuri Simchenko, 1987, personal communication).

In some cases attention has been drawn to the circumstance that such a way of resisting physical strain did not seem to do any harm to the health. "In harvesting hay I can do the work of three men from morning to nightfall without any trouble" (K: Erman, 1833—1848). — "An old woman runs 30 km and does not die"; (Ch: Yuri Simchenko, 1987, personal communication). It seemed to remove all physical and psychic inconveniences: "...he would lay aside his snow-shoes and walk through the deep snow hour after hour...for the mere pleasure of exercise, and without any feeling of fatigue" (Ch: Bogoraz, 1904—1909). — "Under the influence of the fungus it is a trifle to walk 50 km" (K: Vladimir Lebedev, 1987, personal communication). Information about such an effect of AM has been gathered during two centuries and a half already and the existence of this effect of the fungus has also been stressed by the ethnographers Vladimir Lebedev and Yuri Simchenko who are at present studying the problem of AM consumption.

Narcotic purposes

This group includes cases where people thought it necessary to call forth

high spirits. Such were, for instance, feasts on the occasion of neighbours' visits, during weddings or after successful hunting tours where most of the time was passed eating, dancing and singing (K: Krasheninnikov, 1755). Sometimes it also happened in everyday life that pleasant psychic state was induced alone or in company (K: Dittmar, 1897; Ch: Yelena Batyanova, 1987, personal communication; K, Ch: Vladimir Lebedev, Yuri Simchenko, 1987, both in personal communications; kh: Maret Saar, 1987, field work data) and then the time was spent in activities (K: Langsdorf, 1809) or whiled away quietly on one's own (k: Dittmar, 1900; ch: Dittmar, 1900; and Ülo Siimets, 1987, personal communication), or doing both this and that (k: Johelson, 1905, 1908). The best and most pleasant experiences are said to have been gained when the effect of AM began to show itself during sleep (i: Erman, 1833—1848; k: Erman, 1833—1848; Enderli, 1903; ch: Enderli, 1903). Therefore, it was customary to go to bed after consuming AM (i, k: Erman, 1833—1848; K, Ch: Yuri Simchenko, 1987, personal communication).

The expected psychic effect became apparent first and foremost as a positive frame of mind: pleasant feelings (K: Krasheninnikov, 1755; Langsdorf, 1809; Dittmar, 1900; i: Erman, 1833—1848; k: Erman, 1833—1848; Maydell, 1893; ch, k: Bogoraz, 1904—1909; and Vladimir Lebedev, 1987, personal communication), gaiety, wit, humour (K: Krasheninnikov, 1755; k: Enderli, 1903; Bogoraz, 1904—1909; ch: Bogoraz, 1904—1909; s or kh on the Narym: Georgi, 1780), or, in addition to a euphoric state, pleasant visionary or auditory hallucinations (K: Krasheninnikov, 1755; Dittmar, 1900; i: Erman, 1833—1848; k: Erman, 1833—1848; Maydell, 1893; Johelson, 1905, 1908; ch, k: Bogoraz, 1904—1909; and Vladimir Lebedev, 1987, personal communication) and dreams (i: Erman, 1833—1848; k: Erman, 1833—1848; Slyunin, 1900).

An unexpected side-effect might appear in the form of the so-called AM spirit jokes. In hallucinations orders have been given sometimes to perform strange operations alien to common sense, their form being so imperative that they have been carried out to the letter, without question (K: Krasheninnikov, 1755; k, ch: Bogoraz, 1904—1909). At times the agaric eater was impelled to imitate the fungus (k, ch: Bogoraz, 1904—1909).

The depth of psychic changes varied: in some cases the person maintained contacts with his surroundings and could check his behaviour during the whole period when the influence of AM lasted, in others he lost all contacts and control of himself. Some people experienced both states during one spell of AM effect (k, ch: Bogoraz, 1904—1909).

Other conspicuous side-effects often mentioned by the investigators consisted in the magnification of surrounding objects (K: Krasheninnikov, 1755; k: Maydell, 1893; Slyunin, 1900; Johelson, 1905, 1908; Bogoraz, 1904—1909; ch: Mayden, 1893; Bogoraz, 1904—1909) as well as trembling or convulsions of extremities (K: Krasheninnikov, 1755; Langsdorf, 1809; k: Enderli, 1903; Johelson, 1905, 1908).

Attention should be drawn to the circumstance that in authentic sources no case has been observed where the behaviour of the native under the influence of the fungus has proved dangerous to himself or his companions,

but in most of them the positive nature of the psychic state caused by AM has been stressed.

We shall conclude with the case when the Russian ethnographer Vladimir Lebedev (1987, personal communication), during an expedition to the Chukchi, tried the effect of AM on himself.

"The effect was revealed in two hours. I was lying in a tent, talking with two men. At first the topic was interesting for me but, starting from a certain moment, I switched out and felt as if I were watching a widescreen film. The program was conducted by myself. I felt light and merry. At a certain point I resumed the conversation with my companions. Having again lost interest in it, I switched over to my 'film' at the spot where I had parted. I spent two hours in total under the influence of the fungi. During all that time I could wholly retain control of myself; my movements were smooth, I was in a lively and merry mood. I did not detect any side-effects concerning either my spirits or physical condition".

Periodical changes in the psychic state seem to be rather typical of the course of such enjoyments. There are also literary data that the contents of hallucinations as well as dreams could be induced by the person involved.

The use of AM to remove psychophysical fatigue can conventionally also be treated in this group.

"When a hunter goes alone to a far-away forest for a long time and gets tired there, that is to say, when his soul and body are tired out, he will eat dried AM and get kind of drunk. After that he will feel well again and can resume hunting" (kh: Maret Saar, 1987, field work data).

Factors essentially influencing the effect

There are two circumstances which have essential impact: the phase of development of the employed fruit body and the predisposition of the consumer.

Phase of development of the fruit body. The dependence of the AM effect on the phase of development of the fruit body is known in both regions of AM consumption and the problem is treated likewise. The effect of young fruit bodies, i.e. those having small and convex pilei, is regarded as stronger and more efficacious on people than that of older ones with big and flat pilei (kh: Karjalainen, 1927; fn: Lehtisalo, 1924; Ch, K: Yuri Simchenko, 1987, personal communication). The former are used to facilitate work and physical exertions, the latter prove to come in handy to evoke hallucinations or obtain a state similar to that caused by alcoholic drinks (ch: Vladimir Lebedev, 1987, personal communication). The ideas of the Itelmen to this effect have been summed up by Langsdorf (1809) as follows: "The smaller mushrooms, which are bright red and covered with many white warty protuberances, are said to be far stronger in narcotic power than the larger ones, which are pale red and have few white spots".

The Forest Nenets wise men ate mature mushrooms only (Lehtisalo, 1924).

Smaller fruit bodies are also more apt to bring about the desired effect.

"Big fungi are not so obedient as small ones, they may deceive; small fungi are stronger than the big ones but more submissive" (Ch, K: Yuri Simchenko, 1987, personal communication).

The size of the fruit bodies fit for use can be pictured by the following two pieces of information: "The pileus of the fungus must not be bigger than the hollow of the hand with crooked fingers" (kh: Maret Saar, 1987, field work data), "the size is variable, with diameters ranging from 1—1½ to 5—6 inches" (i: Langsdorf, 1809).

The disposition of the consumer. The disposition of the AM consumer functions on two levels. The attitude towards AM seems to be of paramount importance. It depends on the culture of the consumer. This is why the data obtained from experimenters with European manner of thought and from people who have suffered AM poisoning differ from those originating in animist-shamanist cultures. The existence of the proper attitude and the necessity to create it are essential features acknowledged also by the peoples consuming AM. "Only someone who is familiar with the origin of the fly-agaric can eat it 'with fortunate results', but if in his intoxication he does not see the mushroom spirits properly, they may kill him, or he may go astray in the dark" (fn: Lehtisalo, 1924).

The other level consists in materializing one's attitude towards AM by telling the fungus before eating what it is wanted for. This act is known in both regions, it is performed in all cases of AM consumption and cannot be omitted before each single event.

"AM must always be told (either in a loud voice or to oneself) the purpose of its use" (Ch, K: Yuri Simchenko, 1987, personal communication).

"Before eating the mushroom, the man recites over it certain definite formulas stating his wish to see the future, the wish will come true in his dream" (k: Enderli, 1903).

"If the magician eats of the mushroom, this always has a cultic significance, which is only natural, since by eating he creates helpers for himself" (kh, m: Karjalainen, 1927).

Administration of AM

Ways of administration. There have been about fifteen ways of administration. AM has been consumed raw, fried or cooked when fresh, dried, and in the form of an extract or decoction. Sometimes people have drunk the urine of a person intoxicated by AM or eaten the meat of intoxicated reindeer.

In the case of dried fungi either the whole fruit body was eaten, i.e., both the pileus and the stipe (ch, k: Yelena Batyanova, Vladimir Lebedev, 1987, both in personal communications) or the pileus only (kh: Karjalainen, 1927; and Maret Saar, 1987, field work data).

The fungus was swallowed unchewed and washed down with a gulp of water (K, Ch: Steller, 1774; Langsdorf, 1809; and Vladimir Lebedev, 1987, personal communication); the fungus was chewed and swallowed and then

washed down with a gulp of water (kh: Maret Saar, 1987, field work data; k: Maydell, 1893; ch: Bogoraz, 1904—1909); the fungus was masticated and only the saliva swallowed (k: Dittmar, 1900); the fungus was masticated by women but swallowed by men (k: Enderli, 1903; Bogoraz, 1904—1909). The latter way of administration has not been observed in 1970—1980, moreover, people lack any memory of it (Vladimir Lebedev, 1987, personal communication). At times the fungus was eaten with bread (kh: Karjalainen, 1927) or with either butter or fat (kh: Karjalainen, 1927). Sometimes it was eaten after it had been soaked in water for some time (kh: Maret Saar, 1987, field work data).

Dried fungi were also cooked in water for a short period of time and then the liquid drunk either pure (k: Strahlenberg, 1736; Ch, K: Vladimir Lebedev, 1987, personal communication) or mixed with fruit juice (the Russians in Chukotka and Kamchatka: Erman, 1833—1848).

In order to get an extract the fungi were soaked in fruit juice (K: Langsdorf, 1809) or hot water was poured over and left until they became swollen (Ch, K: Vladimir Lebedev, 1987, personal communication). It was known that bog bilberry *Vaccinium uliginosum* strengthened the effect of AM (K: Langsdorf, 1809). An extract was sometimes made with fire weed *Epilobium angustifolium* (K: Krashennnikov, 1755). The remaining fungi were also swallowed (Ch, K: Yuri Simchenko, 1987, personal communication).

The urine of a person intoxicated by AM was drunk either pure (k: Strahlenberg, 1736; Krashennnikov, 1755; Steller, 1774; Langsdorf, 1809; Maydell, 1893; Slyunin, 1900; Enderli, 1903; Johelson, 1905, 1908) or mixed with a beverage (k: Erman, 1833—1848).

The meat of reindeer intoxicated by AM had a psychotropic effect only when the animal was slaughtered in the intoxicated state (k: Steller, 1774; Erman, 1833—1848).

Fresh fungi were eaten either raw (kh or s of the Narym: Georgi, 1780; kh: Karjalainen, 1927) or they were fried or cooked and then eaten as sauce or soup (K: Langsdorf, 1809).

The most widespread manner of AM consumption in both regions was that of eating dried fungi (a review of references of such nature has been given by Wasson, 1968, pp. 153—154; K, Ch: Vladimir Lebedev, Yuri Simchenko, 1987, both in personal communications; kh: Maret Saar, 1987, field work data). It was partially due to the circumstance that drying was the best way of preserving the fungus. The main reason, however, was the idea that fresh fungi were very poisonous (k: Slyunin, 1900). "The poison is then more effective, and kills more speedily" (k: Johelson, 1905, 1908).

The water used to wash down the fungi has been characterized as fresh and cold (k: Slyunin, 1900; K: Steller, 1774; kh: Maret Saar, 1987). "The narcotic effect is also said to be heightened by the drinking of large quantities of cold water afterwards" (K: Langsdorf, 1809).

Dose. The dose depended on the physical state of the individual and could therefore differ greatly (K: Langsdorf, 1809). The maximum dose of dried

fungi, according to literary data, is 21 fruit bodies and even more (Patkanov, 1897). If we do not take into account these doubtful figures (Karjalainen, 1927), all the other literary data and the contemporary ones obtained in personal communication will remain in the interval from $\frac{1}{2}$ to 11 fruit bodies (K: Langsdorf, 1809; and Yuri Simchenko, 1987, personal communication; Ch: Yuri Simchenko, 1987, personal communication; ch: Bogoraz, 1904—1909; and Yelena Batyanova, Vladimir Lebedev, 1987, both in personal communications; k: Krashennnikov, 1755; Erman, 1833—1848; Johelson, 1905, 1908; and Yelena Batyanova, Vladimir Lebedev, 1987, both in personal communications; i: Krashennnikov, 1755; fn: Lehtisalo, 1924; s: Donner, 1933; kh: Karjalainen, 1927; and Maret Saar, 1987, field work data).

In order to call forth hallucinations, 3—11 rather ripe fruit bodies with flat pilei have been used while with the object of facilitating work and other physical strain 3—5 young fruit bodies with convex pilei have been chosen (ch, k: Vladimir Lebedev, 1987, personal communication). There is only one piece of information how the dose was determined in a special case of AM consumption. According to a Khanty of the Yugan, AM was administered until the person's spirits began to rise continuously, i.e. when no falls could be detected (Maret Saar, 1987, field work data). It must be stressed that in that region the fungus was eaten unhurriedly, biting small pieces of the pileus, chewing them carefully, and washing down mouthfuls one by one*.

Data on the quantity of fruit bodies and liquid for making extracts and on the consumed quantities are scanty. For beginners the extract was made of 7—9 fruit bodies per 3 l of water. The effect was expected to show itself after emptying the first mug (K, Ch: Yuri Simchenko, 1987, personal communication). To induce a state necessary for epic performances or to evoke a pleasant psychic state, the extract of three fungi has been used (s or kh of the Narym: Georgi, 1780).

In the case of fresh raw fungi only one fruit body at a time has been eaten and its effect has been compared to that of an extract made of three fungi (Georgi, 1780); three fruit bodies have been regarded as fatal (k: Johelson, 1905, 1908). More fruit bodies could be eaten only when cooked or dried (K: Langsdorf, 1809).

Precautionary measures. No aggressive behaviour has been observed in people of animist-shamanist cultures under the influence of AM. A review of the respective literary data has been presented in the book by Wasson (Wasson, 1968, pp. 155—158, see also pp. 176—178). There have been very few mortal cases (K: Langsdorf, 1809; k: Slyunin, 1900; Enderli, 1903; Johelson,

*Another factor determining the dose was the sacred conception that only an even or uneven number of fruit bodies could be administered. The number depended on certain beliefs. For instance, speaking of the perception of the limits, the Khanty of the Yugan added: "If you feel after eating two fungi that it is time to finish, you should still eat one more". When practising AM consumption one must always take an uneven number of fruit bodies (K, Ch: Yuri Simchenko, 1987, personal communication).

1905, 1908; and Yelena Batyanova, 1987, personal communication; fn: Lehtisalo, 1924). Such a situation was guaranteed by observing several rules.

People apt to aggressive behaviour under the influence of AM were eliminated with the help of the procedure performed with each prospective fly-agaric consumer. The beginners were given a little AM extract, and their behaviour in the state of intoxication was watched. In case of quarrelsome behaviour, the use of AM was prohibited in the future (K, Ch: Vladimir Lebedev, Yuri Simchenko, 1987, both in personal communications)*.

Undesirable course of intoxication was avoided by following three rules.

(1) Weak people consumed AM in the form of an extract, i.e. in the manner where its psychotropic effect was the least (ch, k: Vladimir Lebedev, 1987, personal communication).

(2) The person under the influence of the fungus was not disturbed in any way. "Let him do what he wants to. If the others interfere, he may do himself harm or injure himself" (K, Ch: Yuri Simchenko, 1987, personal communication).

(3) AM and alcoholic drinks were not consumed simultaneously (kh: Kulemzin, 1976; Ch, K: Vladimir Lebedev, Yuri Simchenko, 1987, both in personal communications). "It is said that by using the two together one may even die. The fungus and alcohol have effects contrary to each other. For instance, when two people meet, one of them having eaten AM and the other having drunk vodka, the former sees the latter as being a filthy force, black even in colour, detrimental to men, and he passes by him from afar" (ch, k: Vladimir Lebedev, 1987, personal communication). Slyunin (1900) describes a case where a Koryak intoxicated by AM drank vodka as a wonderful antidote and found the new state most unpleasant.

Effect

The most fundamental reviews of the effect of AM on people have been presented by Langsdorf (1809), Johelson (1905, 1908) and Bogoraz (1904—1909). Watching all the cases of its usage together, they have proceeded from the depth of the changes both physical and psychic, and from a person's stage of contact with the surrounding world. On the basis of the aforesaid, the effect of AM has been classified, distinguishing two or three forms. These classifications do not express all the aspects of AM effect with equal clarity, especially where sacred and magic activities are concerned. This can be explained by the circumstances that investigators of European origin can perceive in full neither the essence of the transfigured state of conscious mind nor the ways of achieving such a state, let alone the differences springing from the use or omission of AM during the rites. Therefore we should estimate the effect of AM according to the explanations of AM consumers themselves.

*Aggressive behaviour, however, comes across very rarely (Vladimir Lebedev, 1987, personal communication).

In addition to the explanations and descriptions given above, we shall review lethal cases of intoxication presented by Karjalainen (1927): "If anyone consumes too much of it, his teeth become clamped together, foam comes out of his mouth, his eyes bulge, and he can be saved only by the forcible administration of milk or salt, for *panx* (AM: M.S.) wants nothing to do with these substances".

Summary

The analysis of AM consumption in animist-shamanist cultures revealed that the fungus has been used as a multifunctional psychotropic substance. Being a motive psychostimulant, it has facilitated work and relieved physical stress. Possessing the ability to speed up the reproduction and processing of information, it has formed part of sacred rites and helped in performances of folklore. It has also been used for narcotic purposes.

The fungus has a simultaneous (but not equal) effect on several psychic functions. It can be directed by the optimum choice of the administered amount as well as proper selection of suitable fruit bodies. The consumer's attitude towards the procedure can also be made a tool to achieve the desired aim.

Possible unfavourable side-effects are eliminated by observing several rules.

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FUNGAL FOES IN YOUR GARDEN

33. ROSE DOWNY MILDEW

The Disease: Rose downy mildew is found virtually everywhere there are roses, but more commonly on glasshouse roses than on those growing outdoors. Irregular rounded bright purplish to reddish brown spots develop on the upper side of the leaves. The under side of the leaf bears the delicate dichotomously branched sporangiophores bearing sporangia. Unlike the more robust mycelium and conidiophores of rose powdery mildew, the fragile sporangiophores can easily be wiped off the leaf. During the later stages of infection, leaves may become necrotic. At this time infection may spread to the calyx, petals and stem. Occasionally the flowers and flower buds are malformed and retarded even when infection of the leaves is slight. In heavy infections young shoots can become so heavily diseased that they die back. Rose downy mildew can be distinguished from rose powdery mildew as the latter appears drier and more powdery due to the abundant conidia.

The Pathogen: *Peronospora sparsa* Berk., can infect wild and cultivated roses (*Rosa californica*, *R. centifolia*, *R. canina*, *R. chinensis* and *R. rubiginosa*), and has also been reported on boysenber-

ry in New Zealand. The sporangiophores normally occur on the underside of the leaves. The air-borne sporangia are generally the most important means of spread, but it has been suggested that the pathogen can also occasionally be spread in seeds. Despite early reports, it seems unlikely that the sporangia form zoospores but germinate directly. It is certain that the mycelium can survive between seasons in a dormant state in rose cuttings as well as rooted plants. Although oospores formed inside the infected host tissue may also enable *P. sparsa* to survive over winter, their role in disease transmission is uncertain.

Control: There is some variation in resistance between *Rosa* hybrids. The damp conditions that encourage rose downy mildew can be avoided by making sure that glasshouses are well ventilated and supplying sufficient heat to avoid condensation. It is possible to control rose downy mildew by some of the broad spectrum general fungicides sold for garden use. It also helps if diseased shoots are cut out during the winter and burnt.

R.T.V. Fox



AMANITA MUSCARIA TAKES ON A TRUSTY NEW ROLE

Despite the popularity of the colourful and highly photogenic *Amanita muscaria*, it has rarely featured in this journal during nearly ten years of colour production (see *Mycologist* 1, p 178 for a photograph and vol 4, p 14 for a painting; it has also appeared in illustrations of stamps). This excellent picture taken by Nick Legon at Denny Wood, New Forest in October 1995 should, therefore, give pleasure to all readers.

Perhaps mycologists are near to saturation point with the exploitation of this fungus in children's publications. Now, an Amanita look-alike has been chosen to accompany Trusty the Hedgehog, the new mascot for the promotion of the National Trust to their juvenile clientele through stationery, badges, soft toys and other merchandise.

The autumn edition of their newsletter *Trust Tracks* will carry a feature on mushrooms that are protected on Trust land.

The Trust delicately reminds us that although a real red and white toadstool does exist, and can be poisonous, Trusty and his fungus are stylised artwork and should not be taken as actual representations.

G Hadley

Isolation and characterization of a general amino acid permease from the ectomycorrhizal fungus *Amanita muscaria*

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SUMMARY

A cDNA coding for a fungal amino acid transporter (*AmAAP1*) was identified from *Amanita muscaria* ectomycorrhizas. The transporter gene was expressed at a basal level under all conditions investigated, but its expression was enhanced 10-fold in the absence of a N source utilized by the fungus. Nitrate was not a suitable N source for *A. muscaria* and resulted in maximal *AmAAP1* expression. The expression of *AmAAP1* in a yeast mutant revealed its function as a high-affinity amino acid transporter with a broad substrate spectrum. AmAAP1 takes up all investigated amino acids with K_m values between 22 μ M for histidine and up to 100 μ M for proline. Gene expression and amino acid uptake data together indicate two main functions for AmAAP1: uptake of amino acids from soil for fungal nutrition, and prevention of an amino acid loss by hyphal leakage in the absence of a suitable N source.

Key words: fungi, basidiomycete, transport, gene expression, N.

INTRODUCTION

Ectomycorrhizas are symbiotic associations of fine roots of mainly woody plants with certain soil fungi (ascomycetes or basidiomycetes). The dominant trees of boreal and temperate forests, members of the Fagaceae and the Pinaceae, are obligate ectomycorrhizal symbionts. It is accepted that in this type of symbiosis, mutual benefit is gained from the exchange of plant-derived carbohydrates for amino acids and mineral nutrients (e.g. phosphate) supplied by the fungus (Marschner & Dell, 1994; Smith & Read, 1997). In nutrient-poor soils, ectomycorrhizal fungi, in collaboration with other soil micro-organisms, are able to exploit organic debris (e.g. litter) as a nutrient source. Some of these nutrients are transferred to the plant (Smith & Read, 1997).

In forest soils, nitrogen (N) is available as nitrate, ammonium or amino acids (in free form or as polypeptides). All ectomycorrhizal fungi investigated so far are able to utilize ammonium as well as free amino acids. The capacity for nitrate uptake is still under discussion (Abuzinadah & Read, 1988; Chalot & Brun, 1998).

We have identified an *A. muscaria* cDNA clone with strong homology to fungal amino acid trans-

porters. Here we present its cDNA sequence and an expression analysis of the gene. The kinetic properties of the corresponding transporter were studied by heterologous expression of the entire cDNA in a yeast mutant.

MATERIALS AND METHODS

Biological material

Amanita muscaria (L. ex Fr.) Hooker strain CS83 was isolated from a fruiting body (Schaeffer *et al.*, 1995). Mycelia were grown on Petri dishes or in liquid culture for 2–24 d (Nehls *et al.*, 1998) with glucose as C source and various additions of amino acids, nitrate or ammonium as N source. The mycelium was collected by filtration with a Büchner funnel under suction, washed twice with 50 ml of water, frozen in liquid N and stored at -80°C .

Mycorrhiza formation was induced with *Picea abies* (L.) Karsten (Schaeffer *et al.*, 1995) and with *Populus tremula* $\times$ *tremuloides* as plant partners (Hampp *et al.*, 1996). Control plants were left without inoculum. Mycorrhizal and non-mycorrhizal short roots were harvested, frozen in liquid N and stored at -80°C .

Mature fruit bodies were collected from the Schönbuch forest near Tübingen, Germany.

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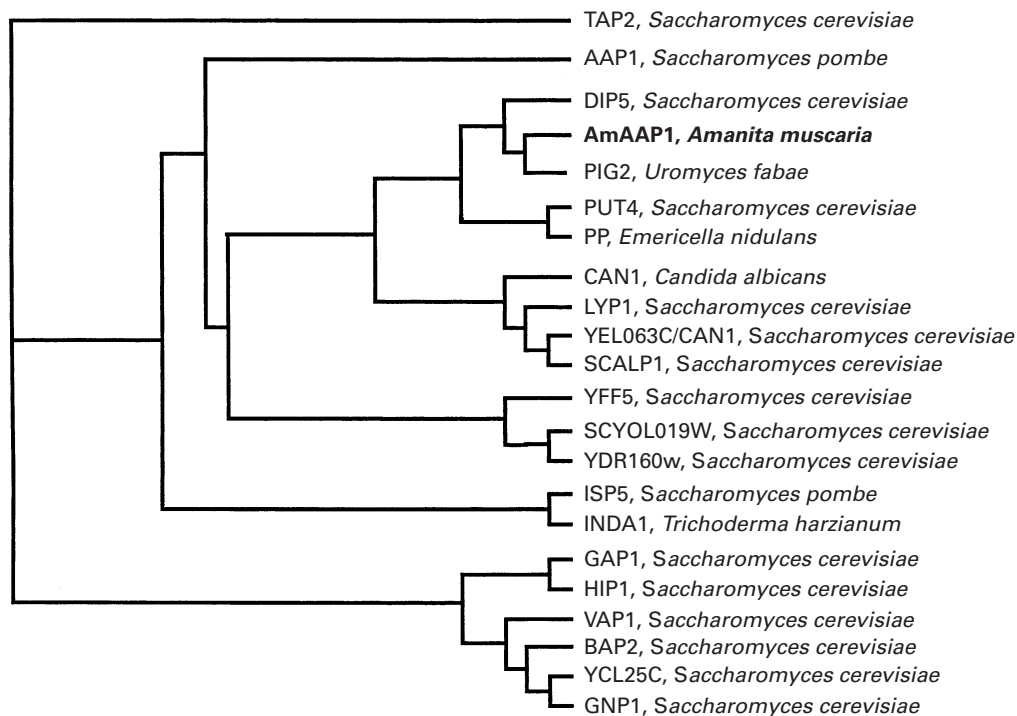


Fig. 1. Dendrogram of a multiple alignment of the deduced protein sequence of *AmAAP1* to other fungal amino acid permeases using ClustalW (version 1.6). Accession numbers: TAP2, P38967; AAP1, D87954; DIP5, X95802; PIG2, U81794; PUT4, M30583; PP, S42708; CAN1, X03784; LYP1, X67315; SCALP1, X74069; YFF5, P43548; SCYOL019W, Z74761; YDR160w, Z50046; ISP5, P40901; INDA1, P34054; GAP1, P19145; HIP1, P06775; VAP1, P38085; BAP2, P38084; YCL25C, P25376; GNP1, U21643.

Northern blot analysis

The RNA of mycorrhizas, fungal hyphae and plant tissues was isolated from 0.2–0.4 g f. wt (Nehls *et al.*, 1998). Northern blots were performed with approx. 5–15 µg of total RNA of non-mycorrhizal lateral roots, mycorrhiza or fungal mycelium (Nehls *et al.*, 1998).

In *P. abies*/*A. muscaria* mycorrhizas total RNA is a 1:1 mixture of both partners (U. Nehls, unpublished). To compare the transcript levels in mycorrhizas with that in fungal mycelia, 15 µg of total RNA was loaded on mycorrhiza lanes, whereas 7.5 µg of total RNA was loaded on lanes of (pure) fungal controls. Fungal RNA was mixed with 7.5 µg of total RNA from sterile *P. abies* roots (which does not hybridize with *AmAAP1* cDNA) to ensure that identical amounts of RNA were loaded on the different lanes.

All northern blot experiments were repeated in triplicate. For quantification of the signal intensities, a flat-bed scanner (Scanmaker IIHR; Microtek, Düsseldorf, Germany) and the program package NIH Image were used (NIH Image is available online from the National Institutes of Health, Germany <http://www.ncbi.nlm.nih.gov>).

Construction of the *AmAAP1* expression vector

The entire *AmAAP1* cDNA was liberated from the pBluescript vector (Stratagene, Heidelberg,

Germany) by double digestion with *EcoRI*/*XhoI*. The cDNA was isolated from an agarose gel with a gel extraction kit (Qiagen, Hilden, Germany) and cloned into the *EcoRI*/*XhoI* double-digested pYPGE15 expression vector (Brunelli & Pall, 1993).

Heterologous expression

The *Saccharomyces cerevisiae* strain JT16 (Tanaka & Fink, 1985) was transformed with the *AmAAP1* expression vector as described by Dohmen *et al.* (1991). For uptake experiments, transformed yeast cells were grown in 0.67% yeast N base (without amino acids), containing 2% glucose and 6 mM histidine, at 30°C with agitation to an attenuation of 0.5 at 600 nm. The cells were centrifuged (10 min at 8000 *g* and 20°C), washed once with distilled water, resuspended in 0.67% yeast N base (without amino acids) containing 2% glucose, and adjusted to an attenuation of 1.2 at 600 nm. Yeast cell suspension (100 µl) was mixed with 100 µl of amino acid solution (from 10 µM to 1 mM containing ¹⁴C-labelled histidine with a specific radioactivity of 40 kBq µ⁻¹) and incubated at 30°C for up to 5 min, with agitation. Sample aliquots of 45 µl each were taken at different times, mixed with 4 ml of ice-cold water and filtered under suction over glass-fibre filters (GF/C; Whatman, Maidstone, U.K.). The filters were washed once with 8 ml of ice-cold water and transferred to 10-ml scintillation vials containing 6 ml of scintillation cocktail (Ultima Gold; Packard,

Groningen, The Netherlands). Radioactivity taken up by the yeast cells was measured in a liquid-scintillation counter (LKB-Pharmacia, Freiburg, Germany).

Miscellaneous

Southern blot analysis was performed as described by Nehls *et al.* (1998). Overlapping sequencing (Sanger *et al.*, 1977) of the entire cDNA was performed by using M13 universal and reverse primers (Stratagene, Heidelberg, Germany) as well as internal primers (MWG-Biotech, Ebersberg, Germany) and the dye-terminator cycle sequencing kit (Amersham).

For the analysis of DNA and protein sequences, the program package GeneJockey II (Biosoft, Cambridge, UK) was used. The sequence data were compared with gene libraries (EMBL and GenBank) by using Blastx (Altschul *et al.*, 1990) and further analysed with Clustal W (Thompson *et al.*, 1994).

RESULTS

cDNA and deduced protein sequences of AmAAP1

A cDNA clone, showing strong homology with other

fungal amino acid transporters, was identified by random sequencing of a *P. tremula* × *tremoloides*/*A. muscaria* ectomycorrhizal cDNA library (U. Nehls, unpublished). This cDNA was used to rescreen the mycorrhizal library. Ten clones out of a total of 140000 clones yielded significant hybridization signals. The longest cDNA clone (accession no. AJ223504) had a length of 1755 bp and contained an open reading frame that could code for a protein of 538 amino acid residues with a molecular mass of 58605 Da. A dendrogram comparing different fungal amino acid permeases with the newly identified *AmAAP1* is shown in Fig. 1. The best homology for the deduced *AmAAP1* protein sequence was obtained with amino acid permeases of *Uromyces fabae* (Hahn *et al.*, 1997) that revealed identities of 47.6% and similarities of 69.9% (Fig. 2).

Copy number of AmAAP1

AmAAP1 is encoded by a single-copy gene in the genome of *A. muscaria* as shown by Southern blot analysis. Digestion of genomic DNA with different restriction enzymes that do not cut within the cDNA of *AmAAP1* (*SalI*, *BamHI* and *XbaI*) showed only single hybridization bands under hybridization con-

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AmAPP1 -----MASEELEKQS--PVYDKPGPEAES----LQGVANDGLSRQLKNRHI
PIG2      MILIQKKDQKNDSEFRIEDELEKHAHQAYYESTEVNASSSPTGDGAVGENTVHRALEQRHI
          :*****: . *:.. :*. * .*. : : * * :***

AmAPP1 AMISIGGVIGTGLFLGTATALRNGGPVGMMLGYALMGLICFCVMISLGEMVAFLEPFGGH
PIG2      SMIAIGGTIGTGLFLGMGEALNKGGLGLILGYMVMGLVVYAMTIALGEMVTMFPVSGSF
          :*:***.***** . **.:***:***:*** :***: :. :*:*****:***.

AmAPP1 IKLAERFVDPAFSFTMGWNYWYNWSIVLPAELSASSVIVGFWNHNINPAVWVVICLVVVV
PIG2      THYPCRFVDPALGFVAVGWNYWYSLTIAVPSEVIAATIVVEYWKAPVNKVWVWITFVLSC
          : . *****.:*:*****. :*:***: *::*: * : : * .***: * :

AmAPP1 TINMLGAGVYGEAEFWFASIKVITITGLIILGIVLDLGGGPNHDLRGFRYWKNPGFVQF
PIG2      GINFFGVRWYGETEFAFAAVKVVAIIVLILVGIVIDIGGGPOTHERIGFRHFIDPGFNFQL
          **: :*. ***:** *:***: * ***:***:*.*****. *:***: : ***** *:

AmAPP1 GGISGTTGQFLGFVAVLTAQAFSYIGTEIVAIAAGETKNPRRNLPRAIKRVIYIRILLSYI
PIG2      HGIPGALGRFLAFWSVFTQASFSYMGTEMVAITAGEAANPRKTVPKAIERVFYRILVFYL
          **.*: *:**.**:*:***:***:***:***:***: ***:.*:***:***: ***: *:

AmAPP1 CGTLIIIGLLVPSNDSGLNLGDGTAASSPFVIAIERAGINSLPHIINACLLTSAWSAASSD
PIG2      GSTLVVGLLVYPYSSPQLLGSTGDASSSPFVIAINRAGITVLPDMINVILLSAISTASSK
          .***:***** ... * . * *:*****:*****. **.:**.* :* ***:***.

AmAPP1 LYTSSRALFGLAVSGNAPKIFLTSSSGLPYVSIAFECMLLRLLAFMATSSGAGK7FGWF
PIG2      LYGGSRVLYGLSKDGMAPRFFSKCNASGLPMWSLLATSSSTA-ALSFMCNLSKAGVAFQWF
          ** .**.*:***. * ***: * . :***** *: . **.*. * **.* **

AmAPP1 ANMTAIAGLMTWFGICVSYIRFYNAKLVQGYDRKTLFSSRLQPFAYFAAGFILVACFF
PIG2      QNLSAMTGILTWWTVLVSIVLFYQGLKVQGLSRDKLHYKAPFQPYASWIALAMLTLMFM
          *:***:***: : ***: **:.***** .*. * :. :*:***: * : : * :

AmAPP1 SGFAVFLKKGWATDTFTVNYLPFVLFPIMYGLARLYYKQGPKPYDEIDLVTGLKEIEQQT
PIG2      SGFETFLKGSWSISDFLANYLTLPIFLGFYLYWKISTRSKFVSSKHVDFTGTREINEMD
          *** .***** *: . *:***: : * : * : : . . .*:*** :*::

AmAPP1 YDEPPNNFLERVWQWVM----
PIG2      AQEREKEIIPTKWKGKFDKLL
          :* : : * . :

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Fig. 2. Sequence alignment of *AmAAP1* with PIG2 of *Uromyces fabae* (Hahn *et al.*, 1997).

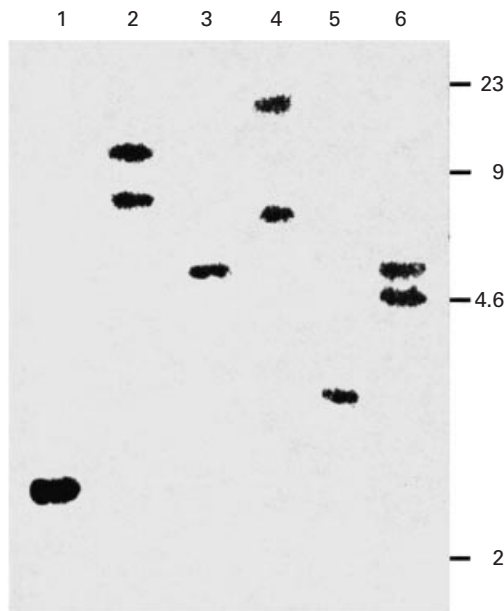


Fig. 3. Determination of the copy number of *AmAAP1*. Genomic DNA was isolated from *Amanita muscaria* mycelia and 15 µg samples of DNA were digested with different restriction enzymes. The DNA fragments were separated on a 0.6% agarose gel, transferred to a nylon membrane and hybridized with a *Bam*HI cDNA fragment of *AmAAP1*. Lane 1, *Sal*I; lane 2, *Eco*RI; lane 3, *Bam*HI; lane 4, *Kpn*I; lane 5, *Xba*I; lane 6, *Hind*III. The positions of molecular mass markers are indicated in kilobases at the right.

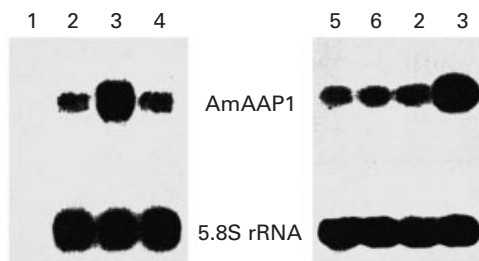


Fig. 4. Nutrition and development-dependent expression of *AmAAP1*. Changes in the transcript level of *AmAAP1* in ectomycorrhizas, fruiting bodies and fungal mycelium grown in liquid culture with 20 mM glucose were compared by northern blot analysis. Total RNA was extracted from non-inoculated *Picea abies* roots (lane 1); *P. abies*/*Amanita muscaria* ectomycorrhizas (lane 2); *A. muscaria* mycelium grown in liquid culture for 3 d in the absence of a N source (lane 3) or in the presence of 1 mM ammonium (lane 4); fruiting bodies (lane 5); or *Populus tremula* × *tremuloides*/*A. muscaria* mycorrhizas (lane 6). RNA was separated on agarose gels, transferred to nylon membranes and hybridized with *AmAAP1* cDNA (top) or *A. muscaria* 5.8S rRNA (bottom).

ditions of high stringency (Fig. 3). This result ensured that in Northern blot analysis, performed under conditions of high stringency, only *AmAAP1* was recognized.

Expression of AmAAP1 under different development and growth conditions

The *AmAAP1* gene was expressed under all conditions investigated. The presence of a N source

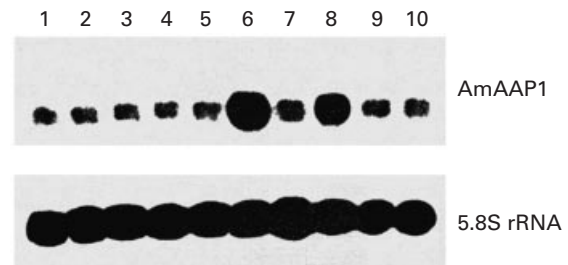


Fig. 5. *AmAAP1* expression in the presence of different amino acids. Fungal mycelia were cultivated in liquid culture in the absence of a N source for 1 wk. Different N sources (2 mM) were added and the mycelium was grown for a further 2 d. Total RNA was extracted, separated on an agarose gel, transferred to a nylon membrane, and hybridized with the *AmAAP1* cDNA (top) or *Amanita muscaria* 5.8S rRNA (bottom). Lane 1, casein hydrolysate; lane 2, ammonium; lane 3, leucine; lane 4, aspartate; lane 5, alanine; lane 6, without amino acids; lane 7, arginine; lane 8, phenylalanine; lane 9, glutamine; lane 10, asparagine.

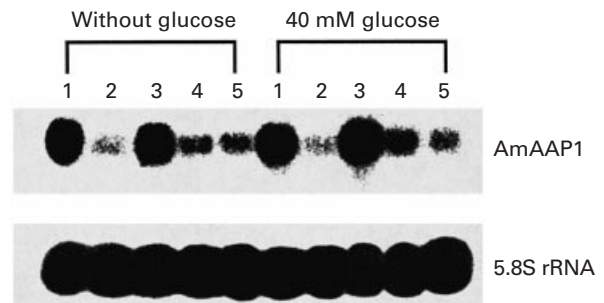


Fig. 6. Effect of glucose, glucose analogues and different N sources on *AmAAP1* expression. Fungal mycelia were cultivated in liquid culture in the absence of a N and carbohydrate source for 1 wk. Different N sources (2 mM) were added and the mycelium was grown in the presence (40 mM) or absence of glucose for a further 2 d. Total RNA was extracted, separated on an agarose gel, transferred to a nylon membrane, and hybridized with the *AmAAP1* cDNA (top) or *Amanita muscaria* 5.8S rRNA (bottom). Lane 1, without N source; lane 2, ammonium; lane 3, nitrate; lane 4, casein hydrolysate; lane 5, ammonium plus nitrate.

that was easily utilized by the fungus (e.g. ammonium and certain amino acids at millimolar concentrations) allowed a basal *AmAAP1* expression (Fig. 4). In the absence of a utilizable N source, or with low N concentrations (in the micromolar range; results not shown) there was a 10-fold enhancement of *AmAAP1* expression. *AmAAP1* was also expressed at a basal level in fruiting bodies of *A. muscaria* (Fig. 4).

There was no difference in the basal level of *AmAAP1* expressed in the presence of ammonium or those amino acids that can be used as a N source (Fig. 5). Only phenylalanine, which is not metabolized by the fungus (Abuzinadah & Read, 1988), led to a strong (10-fold) enhancement of *AmAAP1* expression. Nitrate as sole N source also caused a 10-fold enhancement of *AmAAP1* expression. This indicates that nitrate is not a suitable N source for *A.*

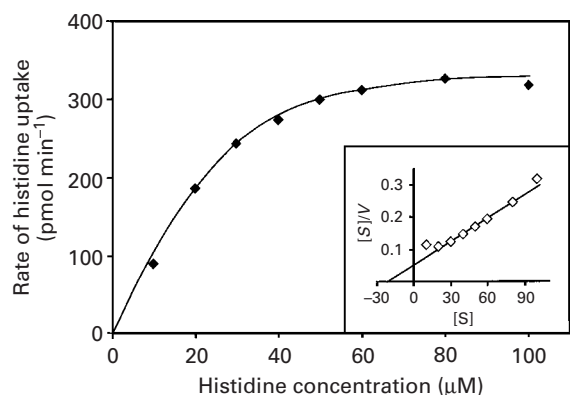


Fig. 7. Uptake of labelled histidine by the *AmAAP1*-expressing yeast mutant. A yeast strain expressing *AmAAP1* was incubated with increasing histidine concentrations (10–100 μM containing the same specific radioactivity (40 $\text{kBq } \mu\text{mol}^{-1}$ [^{14}C]histidine). The rate of histidine uptake (pmol min^{-1}) was calculated from the radioactivity incorporated by yeast cells and used to calculate the K_m value (insert) as described by Hanes (1932).

muscaria. A mixture of ammonium and nitrate resulted in only basal expression of *AmAAP1*, revealing that nitrate also does not act as a signal, enhancing *AmAAP1* expression in *A. muscaria* (Fig. 6). The presence or absence of glucose had no influence on *AmAAP1* expression (Fig. 6).

Heterologous expression of *AmAAP1* in yeast

To investigate whether *AmAAP1* codes for a functional amino acid permease, the entire cDNA was cloned into the yeast expression vector pYPGE15 (Brunelli & Pall, 1993) under the control of the yeast phosphoglycerate kinase promoter. This con-

struct was transformed into the *S. cerevisiae* mutant JT16 lacking both an endogenous histidine uptake system and an enzyme necessary for histidine biosynthesis (Tanaka & Fink, 1985). Only the transformants expressing the *AmAAP1* cDNA in the sense orientation were able to grow on agar plates containing 0.1 mM histidine as sole N source.

The kinetic properties of *AmAAP1* were investigated by the uptake of radioactively labelled histidine (Fig. 7). The K_m for histidine was 22 μM , indicating that this amino acid permease belongs to the high-affinity amino acid uptake systems. Histidine uptake was an active process and could be completely inhibited by addition of the protonophore Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The range of amino acids transported by *AmAAP1*, as well as their transport efficiency, was determined by their competitive effect on the uptake of labelled histidine (Fig. 8). This experiment revealed a clear preference of *AmAAP1* for basic and aromatic amino acids. In contrast, acidic as well as sulphur-containing amino acids and especially proline were taken up with a significantly lower efficiency.

DISCUSSION

The recycling of organic nutrients is an important ecological necessity in mature forest ecosystems, which are usually poor in inorganic nutrients. Because the litter layer of these forests, especially those of conifers, is often acidic ($\text{pH} < 5$), litter mineralization as a result of bacterial activity is limited. Under these conditions, the activity of ectomycorrhizal fungi for litter degradation and the

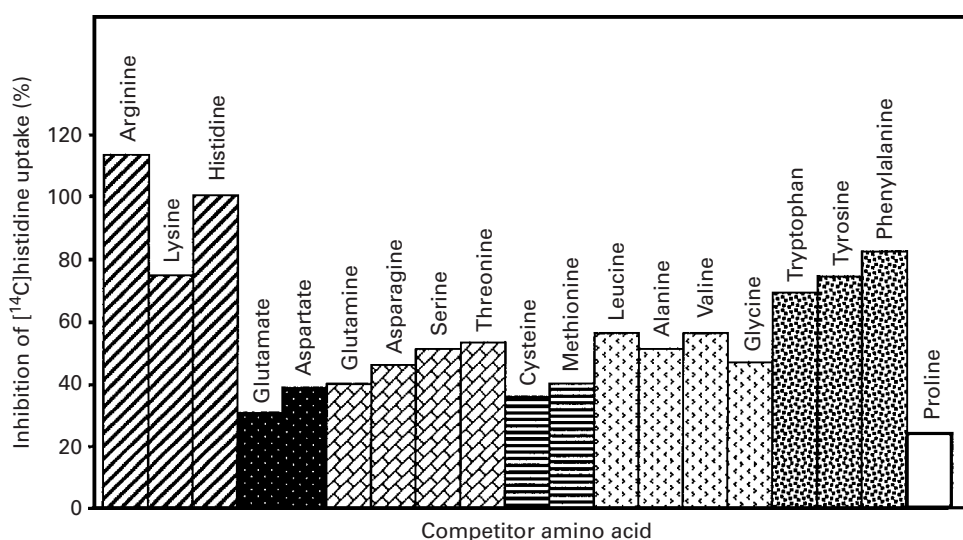


Fig. 8. Uptake efficiency of different amino acids compared with that of histidine. A yeast strain expressing *AmAAP1* was incubated with a mixture of [^{14}C]histidine (25 μM) and an unlabelled amino acid (125 μM). The competition between the uptake of labelled histidine and unlabelled amino acids was measured as a percentage of [^{14}C]histidine uptake. The inhibition of [^{14}C]histidine uptake in the presence of unlabelled histidine was set at 100%.

uptake of organic nutrients from soil is therefore of particular interest for plant nutrition. One aspect of this is the utilization of amino acids as a result of extracellular polypeptide degradation (Abuzinadah & Read, 1986, 1988). Uptake studies with hyphae of the ectomycorrhizal fungus *Paxillus involutus* (Chalot *et al.*, 1996) and mycorrhizas obtained from natural stands (Wallenda & Read, 1999) have revealed the presence of a low-affinity, non-saturable amino acid import system, and a high-affinity, saturable system.

We have cloned an *A. muscaria* cDNA (*AmAAP1*) revealing high homology with amino acid transporters. As shown by heterologous expression in yeast, *AmAAP1* codes for a high-affinity H⁺/amino acid symporter with a broad amino acid spectrum. AmAAP1 has the highest affinity for basic and aromatic amino acids. These amino acids are present in soil (Scheller, 1996) in significantly lower concentrations (8–30 µM) than acidic amino acids or the neutral amino acids alanine and glycine (70–80 µM) which are taken up by AmAAP1 with lower affinity. The K_m values for transport of 22 µM for histidine and of up to approx. 100 µM for proline (as estimated from competition experiments) are therefore in the range of amino acid concentrations found in soil. This makes it quite likely that, in soil, AmAAP1 is involved in amino acid uptake for fungal nutrition.

The presence of amino acids (in millimolar concentrations) that are imported by AmAAP1 and used by *A. muscaria* as N sources (Abuzinadah & Read, 1988), or ammonium that is imported via specific ammonium uptake systems (Marini *et al.*, 1997), resulted in only a low, constitutive expression of *AmAAP1*. In contrast, *AmAAP1* expression was increased 10-fold when hyphae were grown at micromolar concentrations or in the absence of a utilizable N source. Thus, in *A. muscaria*, as in ascomycetes such as yeast or *Aspergillus* (Sophiano-poulou & Diallinas, 1995), amino acid transporter gene expression is regulated at the transcriptional level by N repression.

From the down-regulation of *AmAAP1* expression in the presence of a suitable N source, independently of whether or not it is a substrate of *AmAAP1*, it can be concluded that *AmAAP1* expression is regulated by the internal N status of fungal cells. This assumption is confirmed by studies on *AmAAP1* expression in fruiting bodies. Fruiting bodies, which are supplied with amino acids by soil hyphae and are not exposed to an external N source, expressed *AmAAP1* only at a basal level.

The enhanced expression of *AmAAP1* under conditions of N starvation could also indicate that *AmAAP1* functions in the restriction of amino acid loss by hyphal leakage, in addition to amino acid uptake for nutrition. As shown for a yeast mutant lacking arginine uptake activity, the decreased re-

import capacity for this amino acid resulted in a net loss of arginine from the cells (Grenson, 1973). Thus, enhanced *AmAAP1* expression under N starvation conditions might increase the AmAAP1 content in the plasma membrane of *A. muscaria*, promoting an enhanced re-uptake capacity of fungal hyphae for amino acids.

The regulation of *AmAAP1* expression by the internal N status of the hyphae could also indicate a mechanism that might control the supply of the plant partner with amino acids in symbiosis.

Mycorrhizas are well supplied with N by soil hyphae. The N-dependent repression of amino acid transporter gene expression (as indicated by *AmAAP1*), together with post-translational events that have been described for yeasts (Springael & André, 1999), could therefore result in a decreased fungal re-uptake capacity for amino acids at the plant/fungus interface. In combination with efflux mechanisms this would result in a net export of amino acids.

Nitrate as sole N source resulted in maximal *AmAAP1* expression. It can therefore be suggested that nitrate utilization by *A. muscaria* is impossible or rather poor. In addition, phenylalanine was also not recognized as a N source by the sensing system controlling *AmAAP1* expression. This is in accordance with the inability of *A. muscaria* to grow on nitrate or phenylalanine as N sources (Abuzinadah & Read, 1988).

External C sources had no influence on *AmAAP1* expression. Thus, in contrast with the regulation of a proline permease from *A. nidulans* (Tazebay *et al.*, 1997), C catabolite repression is not involved in the regulation of *AmAAP1* expression. This is in agreement with results obtained for the ectomycorrhizal fungus *P. involutus* (Chalot *et al.*, 1995).

From its expression characteristics and substrate specificity, *AmAAP1* resembles the Gap1 transporter from yeast (Jauniaux & Grenson, 1990). Like *AmAAP1*, *Gap1* codes for a general amino acid permease, and its expression is repressed at the transcriptional level in the presence of a good N source but enhanced in its absence.

In conclusion, we have been able to isolate the first gene coding for an amino acid transporter from an ectomycorrhizal fungus. The uptake characteristics of the encoded transporter protein, as analysed by heterologous expression in yeast, identified AmAAP1 as a high-affinity, general amino acid permease. Gene expression and amino acid uptake data together indicate two main functions for *AmAAP1*: the uptake of amino acids from soil for fungal nutrition, and the prevention of amino acid loss by hyphal leakage in the absence of a suitable N source and thus a low internal N status. Studies on *AmAAP1* give a first insight into amino acid uptake properties and N-dependent gene regulation in *A. muscaria*, an ectomycorrhizal fungus typical of

mature temperate forest ecosystems (Moser, 1978; Rexer *et al.*, 1998).

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Revisiting Wasson's Soma: Exploring the Effects of Preparation on the Chemistry of *Amanita Muscaria*

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Abstract—In 1968 R. Gordon Wasson first proposed his groundbreaking theory identifying Soma, the hallucinogenic sacrament of the Vedas, as the *Amanita muscaria* mushroom. While Wasson's theory has garnered acclaim, it is not without its faults. One omission in Wasson's theory is his failure to explain how pressing and filtering Soma, as described in the Rig Veda, supports his theory of Soma's identity. Several critics have reasoned that such preparation should be unnecessary if equivalent results can be obtained by consuming the raw plant, as is done with other psychoactive mushrooms. In order to address these specific criticisms over 600 anecdotal accounts of *Amanita muscaria* inebriation were collected and analyzed to determine the impact of preparation on *Amanita muscaria*'s effects. The findings of this study demonstrated that the effects of *Amanita muscaria* were related to the type of preparation employed, and that its toxic effects were considerably reduced by preparations that paralleled those described for Soma in the Rig Veda. While unlikely to end debate over the identity of Soma, this study's findings help to solidify the foundation of Wasson's theory, and also to demonstrate the importance of preparation in understanding and uncovering the true identity of Soma.

Keywords—*Amanita muscaria*, hallucinogen, Rig Veda, soma, Wasson

In 1968, R. Gordon Wasson first proposed his groundbreaking theory identifying Soma, the hallucinogenic sacrament of the Vedas, as the classic spotted fairy tale mushroom—*Amanita muscaria*. While Wasson's theory is compelling on many levels he neglected to explain how the pressing and filtering of Soma, as described in the Rig Veda, supported his theory of Soma's identity. This omission has led to several criticisms of his theory, including: (1) that such an elaborate process of extraction and preparation should be unnecessary if Soma were a mushroom; and (2) that no procedure of preparation is known to reliably reduce or eliminate the often unpleasant effects of *Amanita muscaria*. In order to address these specific criticisms over 600 anecdotal accounts of *Amanita muscaria* inebriation

and poisonings were collected and analyzed to determine the impact of preparation on *Amanita muscaria* inebriation. The findings of this study demonstrated that the effects of *Amanita muscaria* were related to the type of preparation employed, and that the often-toxic effects of *Amanita muscaria* were considerably reduced by preparations that paralleled those described for Soma in the Rig Veda. Before addressing this analysis, however, it is important to establish a foundational understanding of the chemistry of *Amanita muscaria*, as it is currently understood, and to understand Wasson's ideas around preparation and the criticisms that have been leveled against this portion of his theory.

The pharmacology of *Amanita muscaria* is not entirely understood. Two primary compounds, ibotenic acid and muscimol, are known to occur in pharmacologically active levels and are partially, if not entirely, responsible for *Amanita muscaria*'s psychoactive effects. Another notable constituent of *Amanita muscaria* is muscarine. Muscarine is a cholinergic agonist which produces significant physiological changes in

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sufficient doses, but is not known to produce psychoactive effects and does not contribute to the psychoactivity of *Amanita muscaria*. Muscazone, a compound related to ibotenic acid, has also been reported from *Amanita muscaria*, but may simply be a by-product of procedures used to isolate ibotenic acid (Catalfomo & Eugster 1970). The psychoactivity of muscazone is unknown, but is considered doubtful (Ott 1993). There is also evidence that the degradation of ibotenic acid results in the production of another, as of yet unidentified, compound (Ott 1976).

Ibotenic acid is the most abundant of the principal compounds, and easily degrades into muscimol through the loss of a carboxyl group. Dehydration of the mushroom is the easiest way to promote decarboxylation of ibotenic acid to muscimol. It has been suggested that heating or cooking may also promote degradation of ibotenic acid due to its low stability (Catalfomo & Eugster 1970). Jonathan Ott (1976) has found that ibotenic acid decarboxylates to muscimol when exposed to acidic conditions, and suggested that ibotenic acid may be converted to muscimol in the acidic environment of the stomach. Interestingly, most ibotenic acid passes through the system unmetabolized and may be reingested in the form of urine to prolong its psychoactive effect. Ibotenic acid is a water soluble compound, but is apparently difficult to dissolve in cold water (Catalfomo & Eugster 1970).

Dr. Scott Chilton (1975), a specialist on mushroom toxins, detailed the following effects from his experiments with pure ibotenic acid: unsteadiness, dizziness, narrowed field of vision, mild visual spasms, muscular twitches, and sleep. Dr. Chilton reported no actual hallucinations from his experiments. According to Chilton, 53 mg was sufficient to produce psychoactive effects, while a dose of 93 mg produced a strong inebriation, including a brief bout of vomiting. Peter G. Waser, a Professor at the University of Zurich, has also reported on his experimentation with a low dose of ibotenic acid. With 20 mg, Waser (1979) reported experiencing flushing, lassitude, and sleep. The psychoactivity of ibotenic acid has been pegged at 1 mg/kg (Ott 1993), making Waser's dose a very light one. It is not clear whether ibotenic acid itself is psychoactive, or whether the psychoactivity is caused purely by the decarboxylation product of ibotenic acid, muscimol (Ott 1993).

Muscimol is five to 10 times more potent than ibotenic acid, and is likely the primary contributor to the psychoactive effects of *Amanita muscaria* intoxication. Muscimol is a salt and is very soluble in water. It is also a thermostable compound, and will not degrade with cooking or boiling. There is some uncertainty as to whether muscimol occurs in fresh specimens of *Amanita muscaria*, or whether it only appears once drying and decarboxylation of ibotenic acid commences (Catalfomo & Eugster 1970). There is still heavy speculation that other, as of yet unidentified, compounds may contribute to the intoxicating properties of *Amanita muscaria*.

Waser (1979: 435), experimenting with 15 mg of muscimol, reported the following effects:

After a phase of stimulation, concentration became more difficult. Vision was altered by endlessly repetitional echopictures of situations a few minutes before . . . I felt sometimes as if I had lost my legs, but never had hallucinations as vivid and colourful as with LSD.

The echopictures experienced by Waser under the influence of muscimol have also been described by others after having ingested *Amanita muscaria* mushrooms, supporting the idea that muscimol is the primary inebriating agent. One individual described his experience as follows:

As I sat on the toilet watching [my son] splash in the tub I had the most peculiar visual experience . . . As I watched him, say laying belly down, my mind captured this image so it remained in my perception, till another motion, say my son sitting upright, caught my attention, of which the new image would appear. The whole effect was sort of a still frame slide show, where an image would remain in perception till a new one burst from the center of the old one (ChemBob 2005).

Other symptoms known to be caused by muscimol include altered auditory and visual perception, visual disturbances, loss of equilibrium and mild muscle twitching. Unlike ibotenic acid, only trace amounts of muscimol pass through the urine unmetabolized (Ott 1993).

Discovered in 1869, muscarine was the first compound isolated from the *Amanita muscaria* mushroom, from which the compound derives its name. It was originally thought to be the active intoxicating agent, a notion that has long been abandoned. Muscarine, however, does produce notable physiological changes, typically characterized by excessive perspiration and salivation, blurring of vision, abdominal pain, nausea, vomiting and diarrhea. These effects usually develop within 15 to 30 minutes after ingestion, but symptoms may take up to an hour to occur in some cases. Muscarine is both water soluble and thermostable, and does not degrade with cooking or heating.

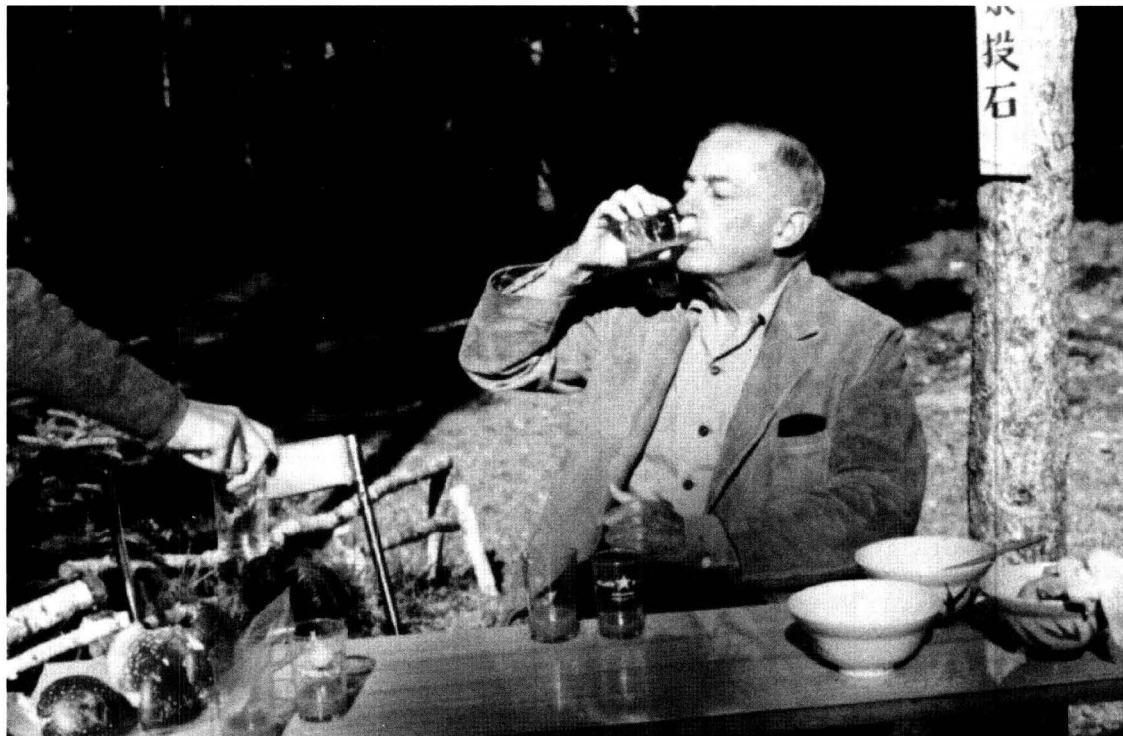
WASSON ON PREPARATION

The Rig Veda speaks of three filters used to purify and prepare Soma for sacramental use. Wasson (1968) has identified these three filters as: (1) a celestial filter, or filter of sunlight; (2) a filter of woolen cloth; and (3) the human body. Wasson focuses predominantly on the first and third filters, providing varying degrees of support for his Soma theory. This section provides an overview of Wasson's thinking on these three filters and also examines the criticisms leveled against this portion of his theory.

The first filter, according to Wasson, is a "celestial filter," which purifies Soma with sunlight. Wasson explained that Soma "catches the sun's rays on its back and holds them there, where they filtre the Soma juice into the plant"

FIGURE 1

Photo of R. Gordon Wasson Sampling the Pressed Juice of *Amanita muscaria* in Japan, circa 1965
(Photo Provided by the Wasson Archives, Courtesy of Robert Forte)



(Wasson 1968: 53). In support of his interpretation Wasson cited several passages from the Rig Veda, including the following:

9.76.4: He [Soma] who has been cleansed by the sun's ray.
(Wasson 1968: 38).

9.86.32: The Soma envelopes himself all around with rays of the sun . . . (Wasson 1968: 54).

Wasson's theory is that the filter of sunlight represents drying or dehydration of the Soma plant, a process that is essential in the preparation of *Amanita muscaria* in Siberia. Among the Kamchadal of the Kamchatka peninsula, it is believed that *Amanita muscaria* that have been sun dried while still in the ground are the most potent, while the Koryak and other Siberian peoples consider fresh *Amanita muscaria* to be toxic (Wasson 1968). These views are likely connected to the change in chemistry of the mushroom through the dehydration process, which increases the strength of the mushroom through decarboxylation of ibotenic acid to the more potent muscimol.

Providing additional support to Wasson's interpretation of the first filter are complementary verses from the Rig Veda that suggest Soma is in a dry state before application of the second filter, when Soma is mixed with water and strained through a woolen cloth. The following passages suggest

that when mixed with water the Soma plant swells in size, a characteristic absent in most fresh plants but familiar to some dried plants, particularly dried mushrooms. Mushrooms are exceptional among plants and vegetables in their ability to reconstitute from a dried state, swelling both significantly and rapidly when soaked in water.

8.9.19: When the swollen stalks were milked like cows with [full] udders . . . (Wasson 1968: 43).

9.74.9: Clarifying Soma, when you are sated with waters your juice runs through the sieve made of wool (Doniger 2005: 123).

10.125.2: I carry the swelling Soma . . . I bestow wealth on the pious sacrificer who presses the Soma and offers the oblation (Doniger 2005: 63).

The second filter is a filter of lamb's wool, used to strain and remove the solids of the Soma plant in order to produce the sacramental beverage. Wasson expended little time examining this filter, stating that it "presents no problems," presumably because this filter is described directly and without any ambiguity (Wasson 1968: 51). Wasson's failure to examine the significance of this stage of the purifying process has led to several criticisms of his work. Professor John Brough (1971: 338) challenged Wasson on this omission, pointing out that "if the Soma-plant had been a mushroom, it would be strange that the elaborate

Vedic process of pounding out and filtering the juice should have been necessary. Why should the plant not have been simply eaten?" David Stophlet Flattery, who proposed that Soma is the plant *Peganum harmala*, also argued that such preparation should be unnecessary if equivalent results can be obtained "by simply chewing the plant materials, as is the case with psychotropic mushrooms" (Flattery & Schwartz 1989: 33).

While Brough and Flattery make a good point, their challenge is based on incorrect assumptions. The first assumption is that *Amanita muscaria* shares similar chemical properties with *Psilocybe* mushrooms, which allow it to be eaten fresh or dried without preparation. The active compounds in *Psilocybe* mushrooms (psilocybin and psilocin), however, have never been isolated from *Amanita muscaria*, do not contribute to its intoxicating effects, and are completely irrelevant to understanding the pharmacology of *Amanita muscaria*. While it is true that *Amanita muscaria*, like *Psilocybe* mushrooms, can produce psychoactive effects when eaten fresh or dried, it does not follow that the pharmacology of the two mushrooms would be similarly affected by the extraction process described for Soma. The second assumption is that the Soma plant cannot simply be eaten, which appears to be based on a bias towards identifying Soma as a woody or fibrous plant (such as *Ephedra* or *Sarcostemma*), which would require water extraction in order to render the drug consumable. Both of these assumptions ignore passages in the Rig Veda that suggest that Soma can be consumed "pressed" or "unpressed." More importantly, however, are indications in the Rig Veda that Soma that has not been pressed is inferior to Soma that has been pressed, suggesting that there is some chemical change that occurs during the extraction process.

6.41.4: Soma when pressed excels the unpressed Soma, better, for one who knows, to give him pleasure (Griffith 1890: 367).

7.26.1: Soma unpressed ne'er gladdened liberal Indra, no juices pressed without a prayer have pleased him (Griffith 1891: 35).

The passages above suggest, regardless of what Soma's true identity is, that pressing and extracting Soma alters the chemistry of the sacrament in some beneficial way. While it has typically been presumed that the "pressing" of Soma is used to extract an active agent from a benign but indigestible plant, hymns describing the ritual preparation of Soma repeatedly speak of Soma being cleansed or purified by the water employed during pressing. For example:

9.62.6: The beautiful plant beloved of the gods, [the Soma] washed in the waters, pressed by the masters, the cows season [it] with milk (Wasson 1968: 28).

9.109.17: The prize-winning Soma has flowed, in a thousand drops cleansed by the waters, mixed with the milk of cows (Wasson 1968: 28).

9.2.5: The ocean [of Soma] has been cleansed in the waters; mainstay of the sky, the Soma in the filtre, he who is favourable to us (Wasson 1968: 47).

The focus on cleansing and purifying Soma through pressing and straining would suggest that Soma is not pressed for the sake of rendering it in a consumable form, as seems to be assumed by Brough, Flattery and others, but rather for eliminating impurities and/or toxic qualities that would otherwise be present in the Soma plant. While Wasson was unable to explain the significance of the second filter as applied to *Amanita muscaria*, this study seeks to illustrate how this step may provide further support for his theory.

The third filter, as proposed by Wasson, is the human body. Wasson advanced his proposition for the third filter by citing the traditions of urine drinking in Siberia and by relying on limited Vedic references to urine, including the following passages from the Rig Veda:

9.74.4: The swollen men piss the flowing [Soma] (Wasson 1968: 29).

8.80.3: In the belly of Indra the inebriating Soma clarifies itself (Wasson 1968: 56).

Wasson went on to explain that "the Soma juice that is drunk by 'Indra' and 'Vayu' in the course of the liturgy is filtered in their organisms and issues forth as sparkling yellow urine, retaining its inebriating virtue but having been purged of its nauseating properties" (Wasson 1968: 55). From a pharmacological perspective Wasson may indeed be correct. While ibotenic acid, one of the mushrooms active constituents, passes in the urine unmetabolized, it is likely that other components of *Amanita muscaria* that contribute to nausea and vomiting, such as muscarine, have been metabolized (filtered) into inactive by-products. This biological process would leave a fairly pure extraction of ibotenic acid in the consumer's urine. Unfortunately, there were an insufficient number of anecdotal reports on urine consumption to provide a proper analysis of the third filter in Wasson's theory.

Pharmacology aside, Wasson's assertion that the third filter is the human body and that mushroom-infused urine is the purest form of Soma has been challenged on two substantial grounds. First is that Wasson's interpretation assumed that "priests appointed to impersonate Indra and Vayu" urinate the intoxicating Soma (Wasson 1968: 30), despite a lack of evidence "in the whole of the Rigveda that priests ever impersonate the gods in any capacity" (Ingalls 1971: 189). The second argument relies on the mere paucity of references to urinating Soma in the Rig Veda, as well as the ambiguity of these rare passages. As noted by one detractor, "the verb to urinate is used in connection with the word soma only twice in the Rig Veda" (Ingalls 1971: 189).

While Wasson's interpretation of the third filter is intriguing, other more feasible explanations present themselves in the text of the Rig Veda. Milk is frequently spoken of as being mixed with pressed Soma or used to further purify Soma, as in the following passages:

TABLE 1
Frequency of Nausea and Vomiting Associated with Different Preparation Types
of *Amanita muscaria* and *pantherina* Mushrooms

Preparation	Nausea	Vomiting	Inebriating
Fresh (n = 41)	49% (20)	37% (15)	81% (33)
Dried (n = 283)	39% (109)	17% (49)	77% (218)
Tea w/ Solids (n = 40)	50% (20)	18% (7)	80% (32)
Tea (n = 94)	31% (29)	7% (7)	79% (74)
Cooked (n = 67)	58% (39)	45% (30)	84% (56)
Total (n = 525)	41% (217)	21% (108)	79% (413)

9.8.5 : When through the filter thou art poured, we clothe thee with a robe of milk to be a gladdening draught for gods (Griffith 1891: 370).
7.21.1: Pressed is the juice divine with milk commingled: thereto hath Indra ever been accustomed (Griffith 1891: 30).

Frequent reference is also made to purifying prayers that were recited over the Soma bowl prior to consumption of the sacred beverage. For example:

9.113.2: Pressed with sacred words, with truth and faith and ardour, O drop of Soma, flow for Indra (Doniger 2005: 133).
9.74.9: Polished by the poets, Soma who brings supreme ecstasy, be sweet for Indra to drink (Doniger 2005: 122).

The addition of milk to Soma and the recitation of prayer during the preparation of Soma are well described steps in the Soma ceremony, as illustrated by the passages above, and either process would make a more plausible candidate for the third filter than recycled urine, as proposed by Wasson.

METHODS

Accounts of *Amanita muscaria* inebriation and poisonings were collected in order to analyze variations in the reports of nausea and vomiting, symptoms often associated with *Amanita muscaria* inebriation/poisoning, by style of preparation. The hypothesis was that each of the first two filters identified by Wasson, the celestial and woolen filters, when applied to *Amanita muscaria* would produce a substantial reduction in the incidence of nausea and vomiting. Such a showing would address criticisms that the elaborate preparation of Soma is unnecessary when applied to *Amanita muscaria*, and that the effects of *Amanita muscaria* are too unpleasant to warrant a sacramental status like that of Soma.

Over 600 accounts of inebriation and poisoning with either *Amanita muscaria* or *Amanita pantherina*¹ were compiled. These accounts were collected from journals (D.D. 1999; Tengu 1998; MN 1996; Salzman et al. 1996; BD 1994; BF 1994; Anonymous 1993; Ott 1976; Pollock 1975), newspaper articles (*Seattle Times* 2006), toxicology reports (Beug 2007, 2006; Cochran 2000, 1999, 1985; Trestrail 1998, 1997, 1996, 1995) and various websites

(Erowid 2009; Lycaeum 2010; Tolento 2008) and forums (Drugs Forum 2010; Entheogen Dot Com 2010; Mycotopia 2010; Shroomery 2010).

The anecdotal accounts of *Amanita muscaria* experiences described a variety of preparation types, including: (1) fresh/raw, (2) dried, (3) tea with solids consumed, (4) tea with solids removed, and (5) cooked. In the fresh category only reports where mushrooms were consumed without preparation (i.e., eaten as is) have been included. The dried category included reports where mushrooms were air or oven dried. The tea with solids consumed category included all water extractions (hot and cold) of dried mushrooms where the mushroom solids were also consumed. The tea category included all water extractions (hot and cold) of dried mushrooms that were strained before consumption. The cooked mushrooms category consisted of reports where mushrooms were prepared by oven or stove, but were not cooked to a dehydrated state. Within each category the reports were examined for frequency of the following symptoms: nausea, vomiting, and inebriation. The resulting differences between each category were then examined through application of logistic regression to determine whether there was a statistically significant relationship between preparation style and effects of the mushroom. Unfortunately, insufficient anecdotal evidence was available on *Amanita muscaria* and urine recycling to analyze the properties of recycled urine in terms of nausea and vomiting.

RESULTS

Considering Table 1, there appear to be some clear trends in the frequency of nausea and vomiting depending on how the mushroom is prepared. Both nausea and vomiting decreased when the mushroom was consumed dried rather than fresh. Vomiting appeared to stay about the same when dried mushrooms were prepared as tea (with solids consumed), but again both nausea and vomiting decreased when the mushroom was consumed as a tea rather than dried or as a tea with solids consumed. While these numbers appeared significant, the difference in population sizes between preparation types made it difficult to come to any conclusions without applying some method of statistical analysis.

The data listed in Table 1 were analyzed using logistic regression to determine whether any of the differences in frequency of nausea and vomiting among preparations were truly significant. The results of this analysis showed that type of preparation was a significant factor in predicting the likelihood of nausea (χ^2 (4, $N = 525$) = 15.19, $p < .01$); and that preparation type was also a significant factor in predicting the likelihood of vomiting (χ^2 (4, $N = 525$) = 39.87, $p < .001$).

While the frequency of nausea appeared to decrease when dried preparations were used instead of fresh preparations, the only preparation type that had a significant effect on the odds of experiencing nausea was tea with solids removed. A preparation of tea reduced the odds of experiencing nausea over a fresh preparation by 53% ($p < .05$). Changes in preparation type had a more substantial effect on the frequency of vomiting experienced during *Amanita muscaria* inebriation; still, only two preparation types had a significant effect on the odds of experiencing vomiting. Eating dried mushrooms reduced the odds of experiencing vomiting over a fresh preparation by 64% ($p < .01$). Drinking a tea also reduced the odds of experiencing vomiting over a fresh preparation by 86% ($p < .001$). While not statistically significant, a preparation of tea (with solids consumed) tended to show reduced odds of experiencing vomiting over a fresh preparation by 63% ($p < .10$).

Finally, a comparison of the different preparation types showed no significant variation in the likelihood of experiencing psychoactive effects (χ^2 (4, $N = 525$) = 1.60, $p > .05$), indicating that while preparation may have influenced the frequency of nausea and vomiting, preparation did not have a similar effect on the production or frequency of psychoactive effects.

DISCUSSION

The change in *Amanita muscaria*'s effects when the mushroom was prepared similarly to Soma, as described in the Rig Veda, was remarkable. Results of the current study showed a significant reduction in the odds of vomiting in consumers of dried preparations as compared to those who consumed the mushrooms fresh. This result serves as an example of how the dehydration process can cause statistically significant changes in the toxic effects of *Amanita muscaria*, and supports Wasson's theory that the first filter used in purifying Soma was a celestial filter, or filter of sunlight. According to the current understanding of *Amanita muscaria*'s chemistry, the biggest difference between fresh and dried *Amanita muscaria* is in the levels of ibotenic acid and muscimol present. Because the drying process decarboxylates ibotenic acid to muscimol, dried preparations have lower concentrations of ibotenic acid than do fresh specimens. This chemical change may account for the differences in the prevalence of vomiting between fresh and dried preparations. The difference in effects between

fresh and dried specimens of *Amanita muscaria* is also supported by the taboo against eating fresh *Amanita muscaria* mushrooms among several Siberian groups with a traditional use of this mushroom (Wasson 1968).

The differences in ill effects between dried preparations and preparations of tea showed that when applying Soma's second filter, a filter of woolen cloth, a significant change in the effects produced by *Amanita muscaria* also occurred. This change could certainly be perceived as beneficial, and could also explain why "Soma unpressed ne'er gladdened liberal Indra" (Griffith 1891: 35). Perhaps unsurprisingly, preparations of the tea in which the solids were consumed shared a similar prevalence of vomiting when compared to dried preparations. This suggested that a component of the mushroom that contributed to the effect of vomiting remained in the mushroom after a hot or cold water extraction. This information seemed to indicate that at least one of the compounds responsible for producing nausea and vomiting was not destroyed by high temperatures (as many preparations were boiled) and was not water soluble. Strained tea still contained ibotenic acid and muscarine, both of which are known to produce symptoms of nausea and vomiting, but the severely toxic components seem to have been removed. Because of the purported difficulty of dissolving ibotenic acid in cold water, a cold water extraction may have lower concentrations of ibotenic acid than hot water extractions, and thus reduce the risk of vomiting even further.

Complicating our understanding of *Amanita muscaria*'s pharmacology, however, is a recent article by William Rubel and mycologist David Arora (2008) that reveals a history of culinary use of this mushroom by scattered populations throughout the world. The most common method of detoxifying this mushroom has been to parboil it in several changes of water, which eliminates all negative and inebriating effects (Rubel & Arora 2008). This would seem to suggest, assuming both methods are valid, that any compound contributing to nausea and vomiting that remains in the mushroom after boiling does not itself produce these results but does so only by acting in concert with *Amanita muscaria*'s water soluble components. More research is needed to determine the specific chemical changes that render water extractions of *Amanita muscaria* less toxic than the dried mushroom.

While there is no indication in the Rig Veda that Soma is cooked, a comparison between fresh and cooked preparations of *Amanita muscaria* has been included in order to address an observation made by Wasson during his unsuccessful experiments with *Amanita muscaria*. Wasson observed one of his colleagues, Rokuya Imazeki, become inebriated after cooking the mushroom caps over an open fire. He and his colleagues were at a loss to explain why Imazeki had become successfully inebriated while the others had not, and could only conclude that cooking had something to do with it (Wasson 1968). While it has been suggested that cooking might cause ibotenic acid to break down due to its instability (Catalfomo & Eugster 1970),

the results here suggested that cooking *Amanita muscaria* did not significantly impact the chemistry of the mushroom as it related to the production of nausea or vomiting. The results also showed that none of the preparation types, including cooking, were significantly more likely to produce psychoactive effects than any other preparation type. This suggested that inebriation may be produced through any of the discussed preparations and that the successful experience of Wasson's colleague was likely a function of dose rather than preparation.

LIMITATIONS

The anecdotal reports used for this study were collected from a variety of sources, and the extent of details provided varied from source to source. As a result, the data used were not entirely uniform and may have been incomplete in some instances. One limitation resulting from the variation in detail is a lack of information on dose and how dose impacted the experiences of nausea, vomiting, and inebriation among preparation types. Without this information it is difficult to gauge how much preparation style, as opposed to dose, contributed to variance in the frequency of nausea and vomiting. Similarities in the frequency of inebriation among preparation types suggested some uniformity of dosage across types, perhaps indicating a limited impact of dose on the analyzed sample. Even with accurate dosage information, however, there remain difficulties in making comparisons among preparations, such as comparing dosages of fresh and dried mushrooms. Direct data collection with uniform questions would help to clarify the impact of preparation versus dose on the occurrence of nausea and vomiting.

The results were also limited by a lack of information on diet and variations in set and setting, factors that may have accounted for some of the differences in reactions among the surveyed reports. Another potential limitation is that no distinction was made between degrees of nausea. As a result,

individuals with mild stomach discomfort were grouped together with those who experienced violent illness. A closer inspection may have shown that preparation also predicted the degree of nausea experienced by the consumer.

CONCLUSION

It has been over 40 years since the publication of Wasson's *Soma: Divine Mushroom of Immortality*, and in that time his theory identifying Soma as the *Amanita muscaria* mushroom has remained one of the most viable proposals to date. One failure of Wasson's, however, was to demonstrate how the preparation of Soma, as described in the Rig Veda, supported his identification of *Amanita muscaria*. The research presented above fills this gap by demonstrating that the effects of *Amanita muscaria* are related to the type of preparation employed, and that the optimal effects of *Amanita muscaria* are produced through preparations that parallel those described for Soma in the Rig Veda. Wasson's theory is further supported by a reading of the Rig Veda that interprets the preparation of Soma as not merely an extraction process, but also a purifying process, since the techniques of Soma preparation when applied to *Amanita muscaria* not only extracted the active principles of the mushroom but also significantly reduced the presence of its nauseating and emetic properties. While this effort in no way closes the door on the debate over the identity of Soma, these findings help to solidify the foundation of Wasson's theory, as well as demonstrate the utmost importance of preparation in understanding and uncovering the true identity of Soma.

NOTE

1. *Amanita pantherina* is a close relative of *Amanita muscaria*, producing the same symptoms as *Amanita muscaria* poisoning and containing the same primary compounds: ibotenic acid, muscimol and muscarine.

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**MUSHROOM POISONING IN INFANTS AND CHILDREN:
THE *AMANITA PANTHERINA*/MUSCARIA GROUP**

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ABSTRACT

The clinical features and management of nine cases of mushroom poisoning due to *Amanita pantherina* (eight cases) and *Amanita muscaria* (one case) admitted to a children's hospital are described. Most ingestions were in the toddler age group with males being more frequently involved. Symptoms occurred between 30-180 min with the onset of central nervous system depression, ataxia, waxing and waning obtundation, hallucinations, intermittent hysteria or hyperkinetic behavior. Vomiting was rare. Seizures or myoclonic twitching occurred in 4/9 patients, but was controlled with standard anticonvulsant therapy. No other anticholinergic or cholinergic signs were prominent. Recovery was rapid and complete in all patients. (Key Words: mushroom poisoning; infants; children; *Amanita pantherina*.)

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"There is probably no area of clinical toxicology more steeped in folklore, misunderstood, or mismanaged than plant and mushroom poisoning." - K. Lampe, Ph.D.(1)

INTRODUCTION

Cases of mushroom poisoning continue to evoke a sense of special concern among nurses and physicians. This is in part due to the mythology surrounding toxic mushrooms and in part to the rarity of serious ingestions, resulting in a lack of familiarity, experience and a general dearth of scientific knowledge.

The overwhelming majority of inadvertent mushroom ingestions occur in children. In 1989, for example, 7560 of 9388 total (80%) cases reported to the American Association of Poison Control Centers National Data Collection System were in children under six years (2). While the symptomatology and management of mushroom poisoning with many of the major groups of toxins is well known and widely available (3), these have been largely based on observations in adults. There are surprisingly few well documented reports of the problem in children. Since there is the possibility that poisoning may differ in children due to differences in ingestion (eg. raw versus cooked), perhaps pharmacokinetics, and even physiological response, we undertook a review of all cases of mushroom poisoning admitted to our hospital (Children's Hospital and Medical Center, Seattle, Washington) over the last decade.

METHODS

All patients admitted to Children's Hospital and Medical Center (Seattle, Washington) with an admitting or discharge diagnosis of mushroom ingestion, poisoning, or intoxication from 1979-1989, were included in the study. Charts were reviewed with special attention being directed to the timing and evolution of signs and symptoms and the modalities of therapy. Two additional children, poisoned in the spring of 1989, but managed at a neighboring hospital were also included. Information regarding these were obtained from the attending physicians.

In all but three cases mushrooms were identified from fragments or complete specimens by Dr. J. Ammirati (Head of Mycology, Department of Botany, University of Washington) or Dr. Lynn Brady (Department of Pharmacology, University of Washington) (Figure 1). In three cases the species were inferred from the descriptions available from parents or other observers, combined with the clinical features. In two of these, spores consistent with the *Amanita* genus were identified in the gastric contents.

For all cases initially managed at other institutions before being transported to Children's Hospital and Medical Center (Seattle, Washington) for admission, copies of the emergency room and transport records were available for review.

Clinical Features

Twelve patients were included in the initial survey of which one male (age 15 years) had suffered from a psilocybe intoxication. The remaining 11 patients had eaten *A pantherina* (10 cases) or *A muscaria* (one case). Their ages ranged from 11 months to 20 years. Nine were boys and three were girls. There was one brother-sister combination. All the poisonings occurred in the spring, from April 6 to May 23. The young children had all ingested mushrooms growing in their yards or immediate neighborhood. A 16 year-old was intentionally seeking an hallucinogenic experience by ingesting a number of *A pantherina* caps. The 20 year-old was a severely retarded, autistic male who was found eating raw mushrooms on the grounds of the institution in which he is cared for. These latter two patients were excluded from further evaluation because of their age.

All the major clinical features are summarized in Table 1. All patients experienced symptoms within 30 to 180 min following the ingestion. Vomiting was unusual, only 3 of 9 having any spontaneous vomiting at the onset of the symptoms. In most patients the initial symptoms and signs were changes in the central nervous system manifested by bizarre behavior or obvious CNS depression. These central nervous system effects were always the most profound aspect of the poisoning and were quite variable between patients. Obtundation of varying intensity was noted in many. Sometimes periods of severe lethargy alternated with periods of agitated abnormal



Figure 1. A collection of typical *A pantherina*.

behavior with screaming, incoherent babbling, confusion, irritability and hallucinations. Generalized tonic/clonic seizures developed in two patients. In two patients seizure-like activity was described, but not witnessed by a physician or nurse. Five patients were noted to have muscle fasciculations or myoclonic jerking.

No significant peripheral anticholinergic signs or peripheral cholinergic signs were described. One patient (Case 2) had minor evidence of both characterized by a modest bradycardia and increased salivation, but mydriasis. Another patient fluctuated between having miotic and dilated pupils.

Treatment

Table 2 lists the treatment received by each patient. Most patients (6 of 9) received syrup of Ipecac within an hour of ingestion, provoking vomiting in all. Both charcoal and gastric lavage were utilized in 6 patients. Diazepam was used in three patients and phenobarbital in two to control seizure activity. Diazepam was used in one patient (Case 2) to manage his hysterical behavior.

TABLE 1

Case No.	Age/Sex	Time to Onset	Symptoms (listed in order of occurrence)	Cholinergic Signs	Anticholinergic Signs
1	4 / F	45	Confusion, lethargy	None	None
2	6 / M	30	Abnormal behavior, delusional, glazed appearance, unresponsive, obtunded, leg-arm fasciculation, sleep alternating with hysteria	Bradycardia, salivation	Mydriasis
3	2 / M	60	Lethargy, myoclonic jerks, hyper-reflexic, variable irritability	-	Mydriasis, Mild HT (132/80)
4	3 / M	NA	Ataxia, stuporous, sluggish, staggering, mild obtundation, incoordination, irregular respiration	Bradycardia, hypotension, myosis	-
5	1 / M	180	Generalized tonic/clonic seizure, obtundation	-	-
6	4 / F	45	Vomiting, abnormal behavior, euphoric, glazed appearance, screaming, ataxia, confusion, irritability, coma-like sleep	-	-
7	2 / F	~ 30	Vomiting, progressive and marked obtundation, later irritable	Bradycardia	-
8	3.5 / M	120	Obtundation, generalized tonic/clonic seizures, severe obtundation, respiratory depression following medication	-	130/86
9	5.5 / M	30	Vomiting, obtundation, ? tonic/clonic seizure, myoclonic twitching, lethargy alternating with agitation, babbling, hallucination	-	Intermittent mydriasis, skin warm and flushed

TABLE 2

Case No.	Ipecac	Lavage	Charcoal	Anticonvulsants	Intubation	Artificial Ventilation	Atropine	Duration of Episode
1	x	x	x	-	-	-	-	4 h
2	x	x	x	Diazepam (5 mg x 2)	-	-	5 doses	9 h
3	x	-	-	-	-	-	1 dose	4 h
4	x	x	x	-	-	-	-	6 h
5	-	x	x	150 mg Phenobarb	For transport	No	-	8 h
6	x	x	-	-	-	-	-	12 h
7	x	-	x	1.25 mg Diazepam	-	-	-	12 h
8	-	-	-	5 mg Diazepam, Paraldehyde, and Phenobarb	x	x	-	14 h
9	-	-	-	2.5 mg Diazepam	-	-	-	12 h

Only one patient (Case 8) had any significant respiratory depression, this following the administration of diazepam, phenobarbital and paraldehyde for seizure control. He was intubated and required assisted ventilation for approximately 8 h. All patients recovered uneventfully within 12 h.

DISCUSSION

While the clinical features of *A muscaria* are very well described (3-5), there are only a few reports of *A pantherina* in either adults or children (4-7). The major toxins are apparently similar being isoxazole derivatives, primarily ibotenic acid and muscimol. However, *A pantherina* often contains additional compounds, such as stizolobic and stizolobinic acid, in concentrations that could be clinically significant (8). These latter compounds are related to L-dopa oxidation products and could produce anticholinergic effects (9).

The age range and sex distribution is rather typical for childhood ingestions and not unexpected. There was one sibling pair (Cases 1 and 2). The older child, a boy, was more severely intoxicated, while the girl was only mildly symptomatic. She did report eating less, but in all cases the estimates of the ingested dose were too imprecise to be reliable. At least in adults as little as one cap has been sufficient to produce a state of intoxication. The time between ingestion and the onset of symptoms was quite variable. In some cases, this may be due to the fact that the children were not observed for a period of time. For example, one mother put her child down for an early afternoon nap about 30 min after the ingestion and discovered him having a seizure two hours later. It is quite likely that he was symptomatic prior to this observation. In general however, most patients displayed symptoms between 30-180 min after ingestion.

In all patients the dominant presenting features involved the central nervous system, with ataxia, obtundation and waxing and waning lethargy-euphoria-hysteria as the most prominent manifestations. While seizures are said to be rare in adults, they are not infrequent in young children. Typical generalized tonic/clonic seizures were observed in two children and seizure-like activity noted in two others. Myoclonic jerking and muscle fasciculation was noted in a number of other patients.

The reasons behind the variable responses between patients to the "same" mushroom is unknown. There are regional differences in the toxin concentrations of mushrooms of the same species and local ecological factors may play an important role. It has been reported that spring and summer mushrooms may contain up to 10 times as much ibotenic acid/muscimol as compared to fall fruitings (10). The state of mushroom hydration and quantity of mushrooms eaten will influence the quantity of toxin ingested. Muscimol has been reported to comprise 0.03-0.1% of the fresh weight of the mushroom (5). Pharmacogenetic or developmental factors may also be important in determining absorption, metabolism, excretion and response to the various compounds.

The inconspicuous nature of either cholinergic or peripheral anticholinergic signs in almost all the patients confirms previous observations about this group of mushrooms. In only one patient (Case 2) was atropine ever warranted, although the heart rate did not drop below 52/min. Physostigmine was never used.

The seizures were easily controlled with either diazepam or phenobarbital. Some authors warn that barbiturates and benzodiazepines may exacerbate the symptoms (11). We were unable to confirm this contention.

From this retrospective study it was not possible to determine the relative efficacy or role of syrup of Ipecac, gastric lavage or charcoal.

The uniformly good outcome in all these patients is well accepted, although the medical and nursing staff were frequently apprehensive. By 12 h after ingestion the children had returned to a normal level of activity. In a few patients the complete lack of concern suggested amnesia for much of the event.

No patient posed a difficult diagnostic problem, since in all but one there was either an observer to the ingestion or the caretaker was sufficiently suspicious to have found the evidence in the yard. However, mushroom ingestion involving the *A muscaria/pantherina* group should be considered in the differential diagnosis of the encephalopathic patient, especially those who show fluctuating symptoms.

The relative rarity of serious consequences of most mushroom ingestions by children is consistent with the local experience in Washington

state as well as nationally. During 1989, the Washington Poison Center network handled 974 calls related to possible ingestions, only five of which resulted in significant effects. This present retrospective study could document only 12 hospitalized children and youth over the decade. Despite these rather optimistic statistics there are occasional deaths and each call needs appropriate attention. Knowledge of the taxonomy, of regional fruiting seasons and likely toxic mushrooms and awareness of the symptomatology of the different toxin groups (3-5,12) is essential.

Based on these cases and the general experience of the Poison Control Center, we would make the following observations and recommendations:

1. If mushroom ingestion is suspected, Syrup of Ipecac should be administered immediately to induce emesis.
2. The child should be observed for at least 3 h by a competent caretaker. This observation can occur at home, with instructions to look for drowsiness, ataxia or abnormal behavior. If no symptoms develop, it is unlikely that significant intoxication with this group of mushrooms has occurred.
3. The first manifestation is usually central nervous system depression with lethargy, progressive obtundation, and ataxia. This may alternate with hyperkinetic behavior. The symptoms have a tendency to wax and wane.
4. Seizures are usually easily controlled with diazepam.
5. Respiratory depression is not a feature of *A pantherina/muscaria* poisoning. If present it is usually iatrogenic due to the anticonvulsant medication. There is usually no indication to intubate these patients.
6. Supportive care and reassurance is normally all that is required. Atropine should be available, but only administered in the very rare event that bradycardia or other cholinergic signs are clinically significant.

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Release and dispersal of basidiospores from *Amanita muscaria* var. *alba* and their infiltration into a residence

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Release and dispersal of basidiospores of *Amanita muscaria* var. *alba* and their potential to infiltrate a nearby residence were investigated. Basidiospore release mainly occurred in the first three days following the expansion of the caps. The concentrations of released basidiospores near basidiomata were 77 137, 75 062, and 41 738 spores m⁻³ in the first three days, respectively, with the highest concentration at 281 738 spores m⁻³ air. After three days, the concentration dropped by 95%. At the second location, airborne basidiospore concentrations dropped 96–99% after three days with the concentrations of 940, 575, and 1359 spores m⁻³ in the first three days, respectively. The diurnal pattern showed a relatively extended night peak. Relative humidity and dew were positively correlated with basidiospore release and short distance dispersal. Rain and rain rate were positively correlated with basidiospore release, but not correlated with short distance dispersal. The basidiospore release period of *Amanita muscaria* var. *alba* was short, but within such a period it released a large amount of basidiospores. However, only less than 5% of basidiospores released were dispersed to the second location 5.2 m away and 2.7 m above the basidiomata. Only <0.1% of basidiospores dispersed from the basidiomata were found inside a nearby residence. *Amanita muscaria* var. *alba* showed a low potential of infiltrating the residence.

INTRODUCTION

Amanita muscaria is a widespread species developing ectomycorrhizas with pine, spruce, fir, larch, birch, and aspen in northern temperate zones and common in Europe, Asia, and North America (Mohan *et al.* 1995, Nehls *et al.* 2001). The var. *alba* is an uncommon variant infrequent in northern North America (Lindgren 1998). *Amanita muscaria* contains ibotenic acid and muscimol, which react with neurotransmitter receptors in the central nervous system (Chilton & Ott 1976, Tupalska-Wilczynska *et al.* 1997, Michelot & Melendez-Howell 2003). A distinctive syndrome including alternating phases of drowsiness and agitation with hallucinations, and sometimes with convulsions will be produced following ingestion of mushrooms of this species (Tupalska-Wilczynska *et al.* 1997). The cap of *A. muscaria* var. *alba* is 4–21 cm diam, convex to plane, white to slightly tan on disc and covered with warts; volva as concentric rings of universal veil tissue on a bulbous base (Lindgren 1998). During the fruiting season, basidiospores released from basidiomata of *Amanita muscaria* varieties could be significant to local

airborne fungal spore composition and populations due to the relative large sizes of the basidiomata.

Basidiospores are one of the major components of airborne fungi in summer and fall in certain areas around the world (Tarlo *et al.* 1979, Vital & Krishnamoorthi 1988, Cosentino *et al.* 1990). Basidiospores were found to be the dominant airborne fungal spores in Hamilton, Ontario (Tarlo *et al.* 1979), and in Connecticut in the summer of 2004 (unpubl. data). However, studies on airborne basidiospores are scarce. Basidiospores in most studies were only identified as a group of basidiomycetes due to the difficulty in identifying basidiospores to genus and family levels based on morphological characters (Li & Kendrick 1995a, b, 1996). Very few studies have been conducted at the genus level for basidiospores. Airborne *Ganoderma*, *Coprinus*, *Agrocybe*, *Calvatia cyathiformis*, *Marasmius rotula* and *Chondrostereum purpureum* were the only few that were investigated (Dye 1974, Gilliam 1975, Tarlo *et al.* 1979, Levetin *et al.* 1992, Li & Kendrick, 1995a, Craig & Levetin 2000, Henriquez *et al.* 2001). Some studies were conducted at the species level on the release and dispersal of basidiomycetes

with a focus of basidiomata which included *Ganoderma applanatum* (Haard & Kramer 1970, Tarlo *et al.* 1979) *Marasmius rotula*, and *Chondrostereum purpureum* (Dye 1974, Gilliam 1975) and *Ganoderma lucidum* in India (Singh *et al.* 1995).

Some basidiospores are allergens (Santilli *et al.* 1985, Levetin *et al.* 1992, Horner *et al.* 1993). Sprenger *et al.* (1988) suggested that basidiospores could be important airborne allergens in the Pacific Northwest. It is necessary to study aerodynamics and temporal patterns of basidiomycetes so that such information can be applied to understanding human exposures and their effects on human allergies. It will be beneficial to study the release and dispersal of basidiospores from basidiomata at the genus and even species levels.

The objectives of the present study were to determine the temporal and spatial characteristics of release and dispersal of basidiospores of *Amanita muscaria* var. *alba* and to elucidate the potential of the basidiospores to infiltrate a nearby residence.

MATERIALS AND METHODS

Two fruit bodies of *Amanita muscaria* var. *alba* in a group developed simultaneously with the stalks touching each other at the base in late September 2004 on the edge of a mixed coniferous and deciduous wood behind a residential property in Avon, Connecticut. The residence, a two-story colonial single family building with walk-out basement and 7 m in height in the front and 10.2 m at the back was 6 m from the basidiomata. The predominant species of the mixed wood are Eastern hemlock (*Tsuga canadensis*), eastern white pine (*Pinus strobes*), American chestnut (*Castanea dentata*), black birch (*Betula lenta*), northern red oak (*Quercus rubra*), white oak (*Q. alba*), and red maple (*Acer rubrum*). These trees are over 20 yr old and much taller than the residences in the neighborhood. The development of the fruit bodies was observed daily commencing from the time of expansion of the basidioma caps. When the fruit body commenced opening, 75 × 25 mm slides coated with a mixture of 90% petrolatum and 10% paraffin wax were put underneath one of the fruit bodies to catch some basidiospores for confirmation of identification. Once the fruit bodies were mature and their caps were fully expanded, air samplers were set up to take samples at 2 h intervals, 12 samples d⁻¹.

Three samplers, Allergenco MK-3 (Environmental Monitoring Systems, Mt Pleasant, SC) were used to take air samples. The Allergenco sampler collects multiple, discrete fungal spore deposits on 75 × 25 mm slides. Twelve samples per day were taken on a slide. All the samples were taken at 15 l min⁻¹ for 10 min. One sampler was posited at ground level and 30 cm away from the two fruit bodies. It was placed on a metal pan to prevent water from soaking and damaging the sampler and to minimize the disturbance caused by outlet air to ground surface. A second sampler was placed at a height of 2.7 m off ground and 5.2 m away

from the fruit bodies (second location) on a deck. The second sampler was 2.4 m away from the residence. The third sampler was placed in the living room of the residence. As the samplers are not waterproof, both samplers outdoors were sheltered by a cover of 62.5 × 45 × 60 cm (Length × Width × Height) from rain damage. Samples were taken from 30 Sept. to 11 Oct. 2004. The slides used for sampling were replaced in the samplers at 18:30 h daily. Once slides were removed from the samplers, all spore deposits were marked with an extra fine sharpie marker at the lower ends of the deposits to allow locating the samples easily for analysis. The single longitudinal traverse method was used to read all 12 samples on a slide.

75 × 25 mm slides coated with a mixture of 90% petrolatum (Fisher, Pittsburgh, PA) and 10% paraffin wax (high melting point 54 °C) were used to collect samples (Li & Kendrick 1996).

After 7 Oct. *Trichoderma harzianum* and *Penicillium* spp. developed on the lower surfaces of the fruit bodies. The fruit bodies collapsed on 12 Oct., and were collected for laboratory examination. They were in too poor a condition to be preserved as vouchers.

Lacto-fuchsin mounting medium and 22 × 30 cover glasses were used to mount the samples. All the samples were analyzed under an Olympus BX 40 compound microscope equipped with phase contrast and DIC optics. The fungal spore identification was conducted at 400× or 1000×.

The sizes of basidiospores were measured by randomly choosing 30 basidiospores from samples collected on 30 Sept., 12 Oct., and prepared from gills of one of the basidiomata, respectively.

Weather data for Avon CT, provided by Rick Bunton, were collected in Avon with a Davis Vantage Pro Weather Station (Davis Instruments, Hayward, CA).

Data were analyzed with NCSS (NCSS, 329 North 1000 East, Kaysville, UT) for Pearson correlation analysis for multiple variables, one-way ANOVA and Tukey-Kramer Multiple-Comparison Test.

RESULTS

Observation of basidiomata development

Two basidiomata in a group started to develop on the ground at the edge of a mixed wood adjacent to a backyard covered by turfgrasses on 23 Sept. 2004. On 26 Sept., the basidiomata were white. Caps were above ground, spherical, and 5–6 cm diam, but not expanded. Separation of caps from volva commenced. The one on the north side was bigger than the one on the south side. On 29 Sept., the basidiomata were partially expanded. Very few basidiospores were released. The basidiomata were fully expanded on 30 Sept. However, the two basidiomata were too close to each other and their caps were forced downward and bent at the sides where the caps met. The basidiomata were similar in

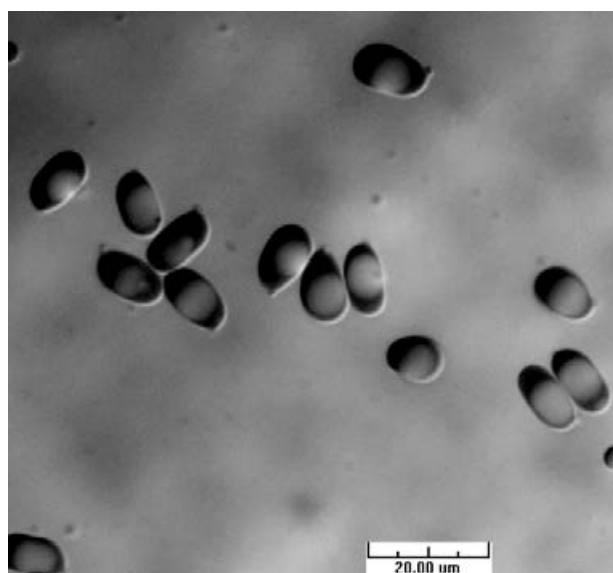


Fig. 1. Basidiospores of *Amanita muscaria* var. *alba* from an air sample.

sizes (13 cm tall, and 12 and 13.5 cm in cap diameter, respectively). On 8 Oct., basidiomata turned from a whitish to tan to light brown and started to shrink. At the same time, *Trichoderma harzianum* and *Penicillium* spp. were observed to develop on the underside surfaces of the caps. Eventually, *Trichoderma harzianum* and *Penicillium* spp. completely covered the underside surfaces of the caps. On 11 Oct., both basidiomata collapsed.

Basidiospores

The basidiospores were broadly ellipsoid to elongate, smooth, thin-walled, hyaline, guttulate, and with a conspicuous hilum (Fig. 1). However, the size of basidiospores at the early stage of spore release was $11\text{--}16 \times 5.5\text{--}8.5 \mu\text{m}$ ($12.5 \pm 2.6 \times 7 \pm 1.4 \mu\text{m}$), significantly longer than the spores released at late stage ($8.5\text{--}13.5 \times 5\text{--}8.5 \mu\text{m}$ ($10.5 \pm 2.2 \times 7 \pm 1.4$)) according to Tukey-Kramer Multiple-Comparison Test ($p=0.05$) (Table 1). The widths of the basidiospores did not show significant differences between the early and late stage of release. There was no significant difference in sizes of basidiospores collected at the early stage of full cap expansion and at the late stage (Table 1).

When basidiomata were partially expanded, very few spores were released. Once the basidiomata were fully expanded, within several hours, a large amount of spores were released into the air. The first three days were the peak period for the basidiomata to release basidiospores. The concentration of basidiospores near the basidiomata in the first day of full expansion of basidiomata was highest with an average of 77 137 spores m^{-3} and the concentration ranged from 21 992 to 151 270 spores m^{-3} (Figs 2–3). However, the highest concentration of 281 738 spores m^{-3} appeared around 2:00 am in the second day with a daily average of 75 062

Table 1. Sizes of basidiospores of *Amanita muscaria* var. *alba* during release and dispersal.

Sampling date	<i>n</i>	Length (μm)	Breadth (μm)	L/B
30 Sept.	30	$13.5 \pm 0.23^{\text{a,b}}$	$7 \pm 0.13 \text{ a}$	$2 \pm 0.04 \text{ a}$
7 Oct.	30	$10.5 \pm 0.23 \text{ b}$	$7 \pm 0.13 \text{ a}$	$1.5 \pm 0.04 \text{ b}$
12 Oct.	30	$10.5 \pm 0.23 \text{ b}$	$7 \pm 0.13 \text{ a}$	$1.5 \pm 0.04 \text{ b}$

^a data are mean $\pm$ SE.

^b Data in the same columns with different letters indicate statistically significant differences according to Tukey-Kramer Multiple-Comparison Test ($P < 0.05$).

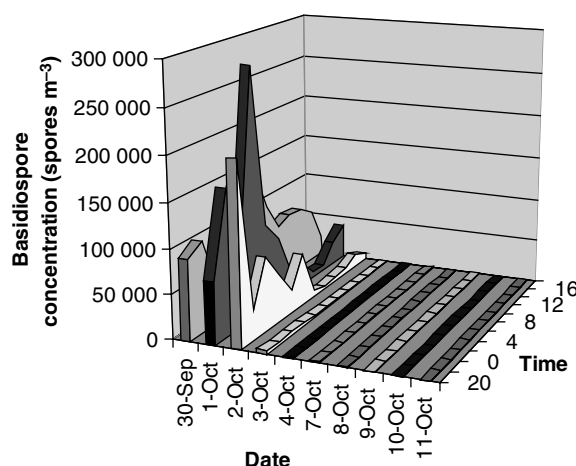


Fig. 2. Temporal patterns of basidiospores of *Amanita muscaria* var. *alba* released from basidiomata.

spores m^{-3} . The average basidiospore concentration in the air in the third day dropped 44% from the second day to 41 738 spores m^{-3} and the concentration ranged from 2261 to 204 838 spores m^{-3} (Fig. 3). The average concentration in the fourth day dropped 95% from the third day to 1954 spores m^{-3} . After 4 d, the basidiospore concentrations were less than 400 spores m^{-3} (Figs 2–3).

Basidiospore release exhibited a diurnal pattern with night peaks according to the means of 10-observation days (Fig. 4). Basidiospores were released mainly during the night with a major peak of 55 562 spores m^{-3} at 02:00 and two minor peaks of 53 071 spores m^{-3} and 41 273 spores m^{-3} at 20.00 h and 06.00 h, respectively. The peak period lasted over 10 h. During the daytime, the concentrations were $< 20\,000$ spores m^{-3} (Fig. 4). The diurnal patterns in the second and third days showed much shorter peak periods (Fig. 2).

Airborne basidiospore concentrations dropped by 96–99% at the second location. Mean concentrations of basidiospores in the first three days were 940, 575, and 1359 spores m^{-3} , respectively (Figs 5–6). Concentrations of airborne basidiospores dropped to < 250 spores m^{-3} three days later following the full expansion of basidiomata (Fig. 6). Airborne basidiospores at this location showed a diurnal pattern, but with a much longer peak period which lasted from

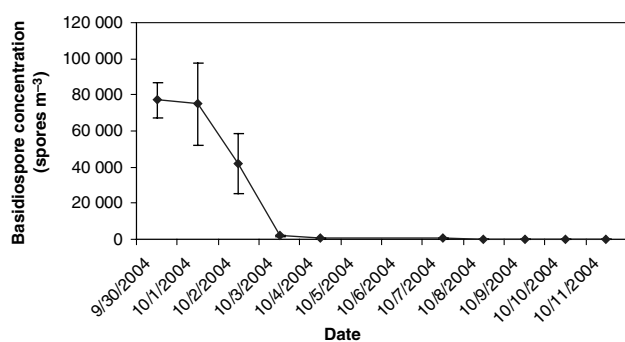


Fig. 3. Daily concentrations of basidiospores of *Amanita muscaria* var. *alba* released from basidiomata. The values are the means of 12-bihourly concentrations ($\pm$ SE).

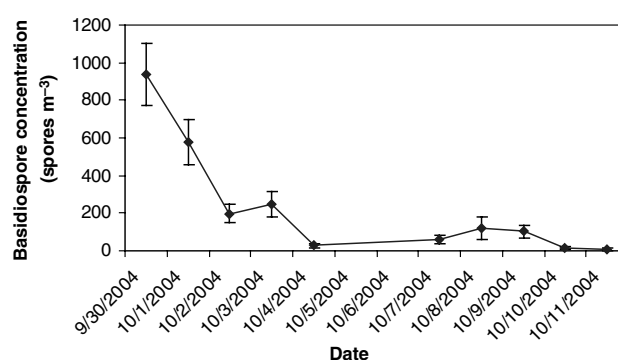


Fig. 6. Daily concentrations of basidiospores of *Amanita muscaria* var. *alba* dispersed to the second location. The values are the means of 12-bihourly concentrations.

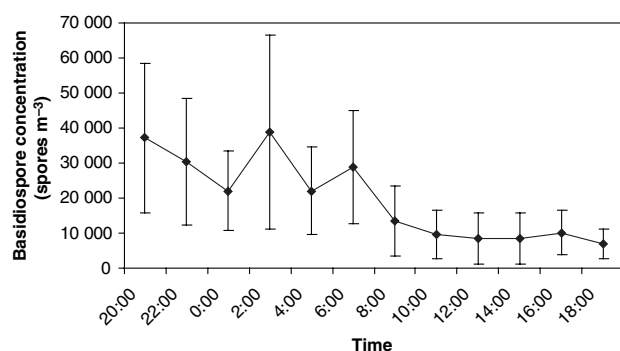


Fig. 4. Diurnal pattern of basidiospores of *Amanita muscaria* var. *alba* released from basidiomata. The values are the means of 10 days ($\pm$ SE).

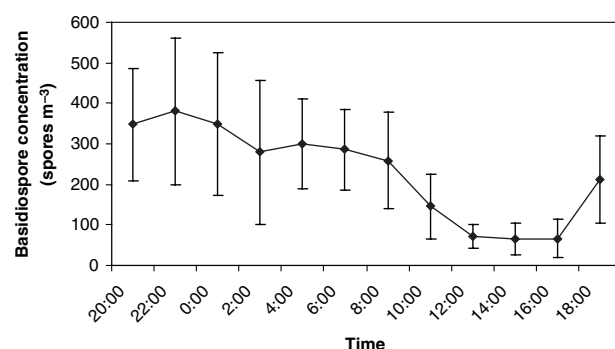


Fig. 7. Diurnal pattern of basidiospores of *Amanita muscaria* var. *alba* dispersed to the second location. The values are the means of 10 days ($\pm$ SE).

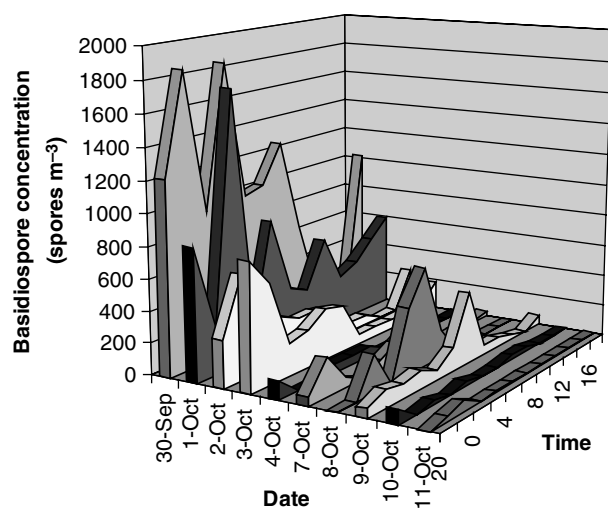


Fig. 5. Temporal patterns of basidiospores of *Amanita muscaria* var. *alba* dispersed to the second location.

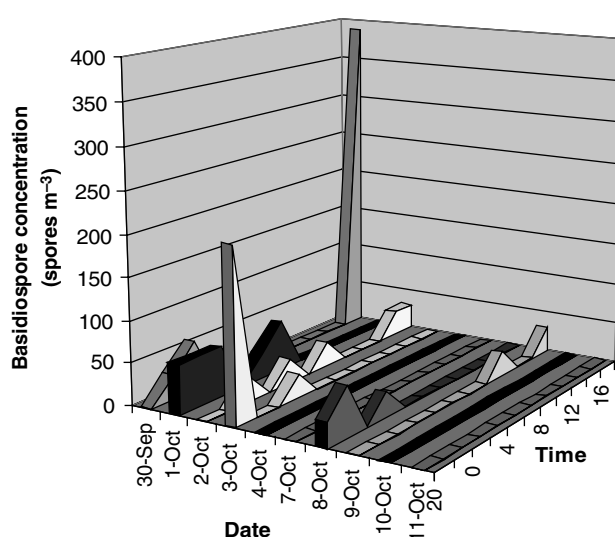


Fig. 8. Daily concentrations of basidiospores of *Amanita muscaria* var. *alba* dispersed into the residence.

18.00 h to 08.00 h (Fig. 7). Airborne basidiospore population was observed to plunge significantly after 10.00 h and reached the lowest level at 14.00 h (Fig. 7).

A very small number of basidiospores infiltrated the residence through open windows or doors from time to time everyday (Fig. 8). Airborne basidiospore

concentrations in the first four days were 40, 22, 12, and 20 spores m⁻³, respectively (Figs. 8–9). After four days, airborne basidiospores were usually absent in the living room, except for 8 and 9 Oct. The highest concentration was 387 spores m⁻³ at 18.00 h on 30 Sept. Airborne basidiospores in the living room showed a

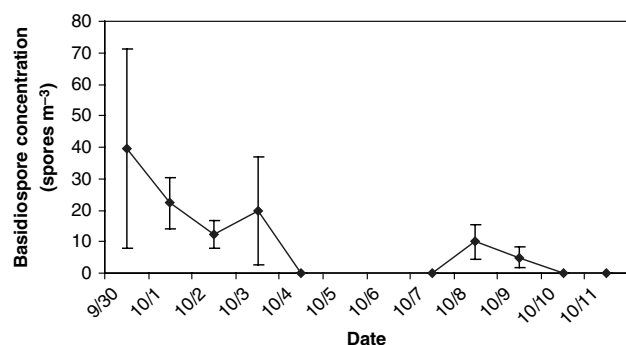


Fig. 9. Daily concentrations of basidiospores of *Amanita muscaria* var. *alba* dispersed into the residence. The values are the means of 12-bihourly concentrations.

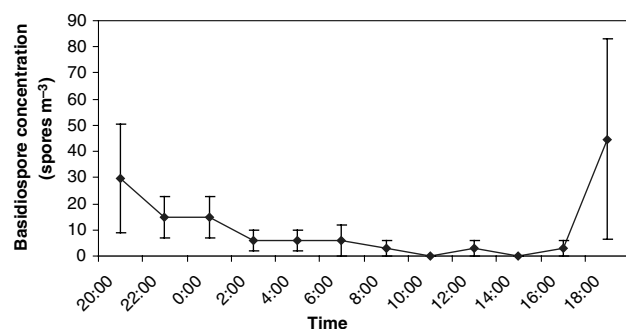


Fig. 10. Diurnal pattern of basidiospores of *Amanita muscaria* var. *alba* dispersed into the residence. The values are the means of 10 days ($\pm$ SE).

diurnal pattern with a maximal period from 18.00 h to 20.00 h and a minimum during the daytime (Fig. 10).

According to Pearson Multiple Correlation Analysis, Airborne basidiospores on the deck (sampler 2) showed a significant correlation with those in the living room (Table 2). Basidiomata age was very strongly and negatively correlated with basidiospore release and short distant dispersal. Time of day was not significantly correlated with basidiospores indoors and outdoors. Keeping windows or doors open was positively correlated with concentration of basidiospores in the living room. However, resident activity did not correlate with basidiospores in the living room. Relative humidity (RH) and dew were positively correlated with basidiospore release and short distance dispersal, while rain (light showers) and rain rate were positively correlated with basidiospore release, but not with short distance dispersal (Table 2). RH, dew, and rain were not correlated with the basidiospores in the living room. Wind, including wind velocity, maximum wind velocity, wind direction and wind run was negatively correlated with basidiospore release and short distance dispersal. Barometric pressure was not significantly correlated with basidiospore release, and short distance dispersal (Table 2). Temperatures (maximum, average, minimum), heat index, heat DD, and THW

(Temperature Humidity Wind Index) were not correlated with either basidiospore release or short distant dispersal (Table 2).

DISCUSSION

Philips (1991) stated that *A. muscaria* var. *alba* developed basidiomata from August to September. However, during the present study, they formed in October in Connecticut. Fully expanded basidiomata of *A. muscaria* var. *alba* lasted over 10 days, but the basidiomata released most basidiospores in the first three days following full expansion. Haard & Kramer (1970) observed that *Inocybe fastigiata* and *Mycena rudilanthiformis* (?*M. roriduliformis*) released their basidiospores mainly in the first 24–48 h after the pileus of the basidiomata were fully expanded. Other agarics and boletes discharged their basidiospore continuously for 1–6 days (Gilliam 1975).

The basidiospores released in the first three days following the full expansion of basidiomata were significantly longer than the ones released several days later and the ones still on gills. Change in length of basidiospores could be in part due to availability of nutrients and water for basidiospore development. One week after the basidiomata's maturation, the basidiomata commenced to shrink and discolour. The deterioration was obvious. Metabolism at this stage in the basidiomata might not be able to provide quality nutrients and enough water for the development of basidiospores. However, more studies are necessary to determine the reasons and mechanism of change in length of basidiospores at different stages of basidioma development. Phillips (1991) described basidiospores that were '7.9–14.1 $\times$ 6.3–9.4 μ m,' shorter and wider than the ones observed (11–16 $\times$ 5.5–8.5 μ m) in this study. Arora (1986) indicated the size of spores of *Amanita muscaria* was 9–13 $\times$ 6.5–9 μ m, however, he did not provide the spore sizes of different varieties. However, in their recent monograph Neville & Poumarat (2004) give the spores of var. *alba* as '(7.7) 9–11(–13) $\times$ (6.3) 7.7–8.3(9) μ m'. These observations were in general agreement with the observation of the spores released at the late stage, 8.5–13.5 $\times$ 5–8.5 μ m in this study, although slightly wider. The differences could be due to variations in fungal populations and geographic distribution.

Based on the literature, there was no indication of the presence of guttules in basidiospores as observed in this study. The presence of guttules could reduce the density of basidiospore and could be beneficial for basidiospore release and dispersal. Guttules could also serve as stored energy source for basidiospores' survival. However, future studies are necessary to understand the role of guttules in the life-cycle of *A. muscaria* var. *alba*.

Amanita muscaria var. *alba* released most of its basidiospores in the first three days following the full

Table 2. Pearson Correlations Analysis of environmental factors and airborne basidiospore populations of *Amanita muscaria* var. *alba* at three locations.

Factor	By basidiomata		On deck		In living room	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
By basidiomata	1.00	0.000*	0.50	0.000*	0.09	0.328
On deck	0.50	0.000*	1.00	0.000*	0.33	0.000*
In living room	0.09	0.328	0.33	0.000*	1.00	0.000*
Basidioma age	−0.56	0.000*	−0.59	0.000*	−0.24	0.007*
Time	−0.04	0.68	−0.04	0.626	0.13	0.145
Open window/door	X ^a	X	X	X	0.18	0.044*
Resident activity	−0.09	0.348	−0.08	0.361	0.16	0.074
Barometric pressure	0.18	0.054	0.16	0.089	0.10	0.263
Dew	0.28	0.002*	0.31	0.000*	0.15	0.094
Heat D D	−0.03	0.738	0.02	0.811	−0.14	0.141
Heat index	0.04	0.645	−0.00	0.926	0.13	0.165
Rain	0.40	0.000*	0.00	0.922	−0.03	0.727
Rain rate	0.39	0.000*	0.01	0.863	−0.02	0.788
Relative humidity	0.33	0.000*	0.44	0.000*	0.03	0.731
Temperature-max	0.01	0.893	−0.04	0.628	0.12	0.191
Temperature-mean	0.01	0.895	−0.05	0.623	0.12	0.200
Temperature-min	0.02	0.857	−0.04	0.676	0.12	0.183
THW	0.04	0.613	−0.00	0.977	0.13	0.159
Wind velocity	−0.29	0.001*	−0.38	0.000*	−0.19	0.035*
Wind velocity-max	−0.32	0.000*	−0.43	0.000*	−0.22	0.018*
Wind direction	−0.19	0.038*	−0.34	0.000*	−0.34	0.000*
Wind run	−0.29	0.001*	−0.38	0.000*	−0.19	0.035*

* *p* is significant.^a X, correlation not biologically meaningful.

expansion of basidiomata. During this period, a very large number of basidiospores were released into the air. The highest concentration of basidiospores near the basidiomata reached 281 738 spores m^{−3}. Gregory *et al.* (1953) observed a similar result with *Serpula lacrymans*, where basidiospore concentrations near basidiomata reached up to 3.6×10^5 spore m^{−3}. In the present study the number of basidiospores released on the fourth day dropped by 95%. This explained why basidioma age was negatively correlated with basidiospores outdoors after the caps of the basidiomata were fully expended. The period of basidiospore release of *A. muscaria* var. *alba* was short and concentrated. Rockett & Kramer (1974) reported that annual lignicolous polypores released large numbers of basidiospores in a single short period, while *Trametes versicolor* and other leathery annual polypores discharged smaller numbers of basidiospore over a much longer period.

Basidiospore release of *A. muscaria* var. *alba* showed a diurnal pattern with a maximum release period at night. However, the pattern was not well defined and peak periods at nights in the first three days varied due to the influence of RH and rain. Part of the reason may have been due to rains and high relative humidity during the release period. There were light showers in the afternoon of 30 Sept., in the morning of 1 Oct., and in the evening of 2 Oct., respectively. Relative humidity was very high ranging from 95 to 100% at night and in part of the morning on both 30 Sept., and 1 Oct. 2004. Rain and relative humidity were important factors for basidiospore release. The effect of rain on airborne

fungus spores depends on its intensity and duration (Lacey 1996). The reason for no correlation between rain and airborne basidiospores at the second sample site may be due, in part, to light showers not affecting short distance dispersal significantly. A number of basidiomycetes, such as *Coprinus* and *Ganoderma*, showed well defined diurnal patterns with a maximum period at night (Li & Kendrick 1995b, Craig & Levetin 2000). Haard & Kramer (1970) found *Crepidotus*, *Panaeolus*, and *Oudemansiella* showed a diurnal pattern with a maximum period around midnight and a minimum during the daytime. However, Kramer & Long (1970) found that under laboratory conditions (21 ° and RH 90%) *Ganoderma applanatum* showed an endogenous rhythm of spore release with maxima occurring at 9–10 h intervals under either alternating 12 h light-dark or continuous light. Gilliam (1975) found that the discharge of basidiospores of *Marasmius rotula* was rain-dependent and did not follow the diurnal patterns displayed by other agarics. These results indicated that the release of basidiospores might be influenced by RH and rain or controlled by an endogenous biological mechanism. The lack of a significant correlation between time and airborne basidiospores outdoors may indicate that the diurnal patterns observed in the present study were not well established or defined. It may not be endogenous as showed by *Ganoderma applanatum*. Further studies are necessary to determine the exact mechanism of controlling basidiospore release and temporal pattern of *A. muscaria* var. *alba*.

Negative correlations between wind speed and basidiospore concentration by the basidiomata and the location which was 5.2 m away and 2.7 m off ground might be due, in part, to the collection efficiencies of the samplers, which might be reduced when wind speed reached certain levels. The lack of correlation between wind direction and basidiospore release was mainly due to basidiomata development at a location where three sides were surrounded by woods; only the east side was open and faced a residential lawn. At the same time, ground cover plants could interfere with air movement on the surface of the ground. However, air could move freely around the sampler which was 5.2 m away from the basidiomata. The negative correlation might be due to the local turbulence caused by the rail around the deck. As a result, the collection efficiency of the sampler may be reduced.

The concentrations of airborne basidiospores at the second location dropped 98%. The results indicated that 98% of basidiospores settled within an area with a 5.2 m radius and fewer than 2% of basidiospores dispersed to areas beyond 5.2 m from the basidiomata and 2.7 m above them. Philip H. Gregory and Margaret F. Gregory predicted that 90% of fungal spores from sources near the ground would deposit within 100 m in normal turbulence (Lacey 1996). The dispersal distance of the basidiospores of *A. muscaria* var. *alba* was much shorter in the present study. Part of the reason may be that the spore dispersal process is complex. Not all spores will travel the same distance due to wind turbulence or irregular air movement (Lacey 1996). McCartney (1991) indicated that the spore dispersal process is determined by wind speed, turbulence, and spore size. The size (relative large) and shape (broadly ellipsoid to elongate) of basidiospores of *A. muscaria* var. *alba* may be the two major factors in their rapid deposition. Low wind speeds and lack of eddy near the ground could be another reason also. However, further studies on spore dispersal of this fungus are necessary.

Airborne fungal spores can cause not only allergenic reactions, but also infections and mycotoxicosis to human beings and animals (Lacey 1996). It is clear that basidiomata of *A. muscaria* var. *alba* can release a large number of basidiospores into the air at its peak period. However, it is not fully understood when mushroom hunters or individuals within the close proximity of basidiomata are exposed to such a high concentration, what kind of health effects these basidiospores may have, especially for the species which are toxigenic. Also, it will be useful to determine how many basidiospores can be developed in a basidioma in future studies.

As the caps of the basidiomata were not completely expanded and part of caps bent downward, release and dispersal of basidiospores from these parts of caps might be significantly reduced.

Only a very small number of basidiospores (<0.001%) released from the basidiomata were recovered inside the residence living room, even in close

proximity to the basidiomata and with open windows or doors from time to time. This indicates that *A. muscaria* var. *alba* has a low dispersal ability and its ability to infiltrate a residency is relatively low. The reason for the lack of correlation between resident activity and airborne basidiospores was due to, in part, the relatively large size of the basidiospores. Routine resident activity may not be efficient enough to re-suspend settled basidiospores into the air. The filter in the HVAC system in the house may play a significant part by efficiently removing a large amount of basidiospores infiltrating the residence from the indoor air. Further studies on fungal infiltration of residences and other buildings are necessary to determine the interactive effects with architecture, building materials, HVAC systems, and maintenance and subsequently to determine the functional relationships of airborne fungi indoors and outdoors. Information from such research is imperative for the professionals who are conducting indoor air quality and indoor fungi investigation and remediation.

The rain shields used to protect the samplers might have effects on the collection of basidiospores. Rain might affect both basidiospore release and dispersal. Rain falling on the caps would generate minute vibrations, which might directly affect the release of basidiospores. Rain could wash out airborne basidiospores from the air and reduce the dispersal distance of this fungus. At the same time, the rain shield might significantly change the aerodynamics of air movement around the samplers. Such a change might have a significant effect on spore collection efficiency. Thus, it will be necessary to study the effects of rain shield on spore collection. It will be beneficial if the manufacturer can develop a water proof model for aeromycological research.

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Phylogeographic Analyses of a Boreal-Temperate Ectomycorrhizal Basidiomycete, *Amanita muscaria*, Suggest Forest Refugia in Alaska During the Last Glacial Maximum

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Abstract Beringia, including Alaska and North-Eastern Siberia, has long been a focal point for biogeographical research in a wide range of plant and animal taxa. Whether boreal forest refugia existed in Beringia during the Last Glacial Maximum (LGM) is a major but unanswered question in Quaternary science. We analysed DNA sequence data from *Amanita muscaria* using phylogenetic and coalescent methods to test whether this boreal-temperate ectomycorrhizal fungus was present in Alaska during the LGM. Our data indicates that there are at least two endemic groups showing no detectable migration from or to populations that survived the LGM in Southern refugia. One of these occurs in the boreal forests of Interior Alaska, while the other group is endemic to the maritime rainforests from Southeast Alaska to the Olympic peninsula. These results suggest genetic isolation preceding the LGM and support the hypothesis of glacial forest refugia in Interior Alaska and along the coast of Southeast Alaska and British Columbia.

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1 Introduction

Climatic and geological changes in the Tertiary and Quaternary dramatically influenced the distribution of flora and fauna in North America. In the Tertiary, North America became separated from Eurasia, resulting in vicariant populations of many species of the ancient flora and fauna (Budantsev 1992; Graham 1999). During glacial maxima, plants, fungi and animals were forced to unglaciated refugia, from where they expanded to newly exposed areas in warmer interglacial periods. Reconstruction of these historical events is of paramount importance because they had major influences on past speciation events and are reflected in present day population structures.

Beringia, including Alaska and North-Eastern Siberia, has long been a focal point for biogeographical research in a wide range of plant and animal taxa. This high level of interest arises for two principal reasons. First, due to its diverse landscape and climate and the fact that much of the region remained ice-free during glacial maxima, Beringia served as a refugium for Arctic and subarctic flora and fauna (Adams and Faure 1997; Brubaker et al. 2005; Edwards et al. 2000; Hultén 1968). Second, during much of the Tertiary and the Quaternary periods, Beringia was the major land connection between Asia and North America and provided migration routes to a wide variety of organisms (for example, see Elias et al. 2000; Qian 1999; Swanson 2003). High genetic diversity and evidence for glacial refugia in Beringia have been reported in several, predominantly arctic-alpine animal and plant taxa, such as the Tundra Vole (*Microtus oeconomus*) (Brunhoff et al. 2003), Bighorn Sheep (*Ovis canadensis*) and Dall Sheep (*O. dalli*) (Loehr et al. 2005), Columbian Ground Squirrel (*Spermophilus columbianus*) (MacNeil and Strobeck 1987), Old World Swallowtail (*Papilio machaon*) (Sperling and Harrison 1994), *Amara alpina*, an arctic-alpine ground beetle (Reiss et al. 1999), *Paranoplocephala arctica*, a parasitic cestode of collared lemmings (Wickström et al. 2003), Entire-leaved Avens (*Dryas integrifolia*) (Tremblay and Schoen 1999), Purple Saxifrage (*Saxifraga oppositifolia*) (Abbott and Comes 2003), Arctic Bell-heather (*Cassiope tetragona*) (Eidesen et al. 2007) and the Bog Blueberry (*Vaccinium uliginosum*) (Alsos et al. 2005).

Whether fragments of boreal forest existed in Beringia during the Last Glacial Maximum (LGM) is a major, but, as yet, unanswered question in Quaternary science. Although earlier pollen-based biome reconstructions suggest that all of Beringia was covered by arctic tundra with no conifers (Edwards et al. 2000; Kaufman et al. 2004; Swanson 2003), phylogeographic analyses of DNA sequences and the most recent pollen data support the theory that *Picea glauca* and *P. mariana* existed in small refugia in Alaska (Anderson et al. 2006; Brubaker et al. 2005).

Ectomycorrhizal (ECM) fungi are obligate root-associated mutualistic symbionts of trees in boreal, temperate and tropical forests (Smith and Read 1997; Bas 1969; Härkönen et al. 1994; Simmons et al. 2002) and their past and present populations likely have been shaped by the distribution of their host trees. The likely importance of host trees in the distribution of ECM fungi has been repeatedly noted.

However, despite their ecological importance, very little is known about ECM phylogeography. Furthermore, learning about the phylogeographic history of ECM taxa not only provides information related to the organism in question, but may also help to resolve whether or not their hosts survived the LGM in Beringian refugia. To test the hypothesis of persistent LGM forest refugia in Alaska, we analysed molecular data from *Amanita muscaria*, an ECM fungus native to temperate and boreal forest regions of the Northern Hemisphere.

2 Materials and Methods

Multi-locus DNA sequence data for *A. muscaria* specimens were generated in previous studies (Geml et al. 2006, 2008, and Oda et al. 2004). Geml et al. (2006) reported that there are at least three phylogenetic species clades within the *A. muscaria* species complex that occur in Alaska (referred to as Clades I, II, and III). To our knowledge, Clade I is restricted to North America, while Clades II and III occur in Eurasia and in Alaska. For our tests in this study, 114 ITS rDNA sequences were chosen, which represent the three phylogenetic species. Because the phylogenetic species mentioned are non-interbreeding entities, population-level analyses were conducted separately for each species clade. Multiple sequence alignments were made using Clustal W (Thompson et al. 1997) and subsequently were corrected manually. Identical sequences were collapsed into haplotypes using SNAP Map (Aylor and Carbone 2003) after recoding insertion or deletions (indels) and excluding infinite-sites violations. The analyses presented here assume an infinite sites model, under which a polymorphic site is caused by exactly one mutation and there can be no more than two bases segregating. Base substitutions were categorised as phylogenetically uninformative or informative, and as transitions or transversions. Site compatibility matrices were generated from each haplotype dataset using SNAP Clade and SNAP Matrix (Markwordt et al. 2003) to examine compatibility/incompatibility among all variable sites, with four resultant incompatible sites removed from the data set. This was important as subsequent coalescent analyses assume that all variable sites are fully compatible. Two migration models were used. First, MDIV (Nielsen and Wakeley 2001) was used to determine whether there was any evidence of migration between Alaskan and non-Alaskan populations in each species clade, i.e. to test whether Alaskan populations could have survived the LGM in local forest refugia. For this purpose, specimens were assigned either to the 'Alaskan' or the 'non-Alaskan' group based on their localities. In Clades I and II, the 'non-Alaskan' groups consisted of specimens collected in the contiguous states of the US or in Eurasia, respectively. These represented populations that survived the LGM in Southern refugia. MDIV implements both likelihood and Bayesian methods using Markov chain Monte Carlo (MCMC) coalescent simulations to estimate the migration rate (M), population mean mutation rate (Θ), divergence time (T) and the time since the most recent common ancestor (TMRCA). This approach assumes that all populations descended from one panmictic population

that may or may not have been followed by migration. For each dataset, the data was simulated assuming an infinite sites model with uniform prior. We used 2,000,000 steps in the chain for estimating the posterior probability distribution and an initial 500,000 steps to ensure that enough genealogies were simulated before approximating the posterior distribution. Second, if MDIV showed evidence of migration, MIGRATE was used to estimate migration rates assuming equilibrium migration rates (symmetrical or asymmetrical) in the history of the populations (Beerli and Felsenstein 2001). We applied the following specifications for the MIGRATE maximum-likelihood analyses: M and Θ generated from the F_{ST} calculation, migration model with variable Θ and constant mutation rate. Subsequently, we reconstructed the genealogy with the highest root probability, the ages of mutations and the TMRCA of the sample using coalescent simulations in Genetree v. 9.0 (Griffiths and Tavaré 1994). Ages were measured in coalescent units of $2N$, where N is the population size.

3 Results

Two of the three species clades (Clades I and II) possessed sufficient variation in the ITS to test whether the Alaskan populations were genetically differentiated from non-Alaskan populations. The third, Clade III, was only represented by ten individuals forming three haplotypes with minimal variation. In this species, all Alaskan specimens carried the most frequent haplotype that was shared with Asian specimens as well. The datasets consisted of 653 and 640 characters for Clade I and Clade II, respectively, including gaps.

In Clade I, the ‘North American’ clade, 15 haplotypes were found, of which only one, haplotype D, was found in Alaska (Tables 1 and 2). MDIV showed no evidence of gene flow ($M \sim 0$) and estimated a divergence time of $T \sim 0.9$ between the Alaskan and non-Alaskan populations (Fig. 1a). Therefore, in simulations using Genetree, we assumed subdivided population structure and a very low level of migration (close to 0) for the starting backward migration matrix. The coalescent-based genealogy was informative with respect to the resolution of the splitting event between the Alaskan and non-Alaskan populations and inference of the mutational history with respect to variation between and within populations (Fig. 2a).

In Clade II, the ‘Eurasian’ clade, 14 haplotypes were found, six of which occur in Alaska (Tables 1-2). Of these six, five were found only in Alaska (haplotypes C, D, G, H, I), while one (haplotype A) represented the most abundant haplotype in the sample, spanning Eurasia and Alaska. It must be noted, that haplotype D (or subclade II/A in Geml et al. 2006), a lineage endemic to the maritime rainforests of the Pacific Northwest, was considered ‘Alaskan’ as opposed to ‘Eurasian’, despite not being strictly restricted by the political boundaries and occurring as far South as Northwestern Washington state. Apart from this, no other Clade II haplotypes have been found in North America, outside Alaska. Despite the very similar values for Θ (Fig. 1), the population structure in Clade II proved to be very different from

Table 1 Identity of *A. muscaria* haplotypes in our sample. Haplotype letters correspond to those in Table 2 and Fig. 2

Haplotypes	Specimen	Geographic origin
Clade I		
A	45785	Hampshire, Massachusetts, USA
B	45820	Bronx, New York, USA
C	CMP3143	Cochise Co., Arizona, USA
C	RET145-2	Tlaxcala, Mexico
C	CMP0648	Cochise Co., Arizona, USA
C	RET144-10	Tlaxcala, Mexico
D	GAL15330	Fairbanks, Alaska, USA
D	GAL16775	Fairbanks, Alaska, USA
D	GAL15776	Bonanza Creek LTER site, Alaska, USA
D	GAL16654	Fairbanks, Alaska, USA
D	GAL15336	Fairbanks, Alaska, USA
D	GAL17984	Fairbanks, Alaska, USA
D	GAL17982	Fairbanks, Alaska, USA
D	GAL15335	Fairbanks, Alaska, USA
E	RET145-1	Tlaxcala, Mexico
F	CMP1345	Cochise Co., Arizona, USA
G	49100	Cascade, Idaho, USA
H	45840	Lawrence, Massachusetts, USA
I	45843	Hampshire, Massachusetts, USA
J	45060	Amador, California, USA
J	LG382	Santa Cruz Island, California, USA
K	44761	Alpine, California, USA
L	45863	Mendocino, California, USA
L	RET320-1	Fremont Co., Idaho, USA
L	LG882	Santa Cruz Island, California, USA
M	RET271-2	Sussex Co., New Jersey, USA
N	45883	Piscataquis, Massachusetts, USA
N	RET271-3	Somerset Co., New Jersey, USA
O	RET383-3	Newfoundland, Canada
O	RET124-2	Suffolk Co., Massachusetts, USA
O	RET338-9	Clallam Co., Washington, USA
O	RET289-3	Cape May Co., New Jersey, USA
O	RET158-7	Burlington Co., New Jersey, USA
O	RET303-4	Monmouth Co., New Jersey, USA
Clade II		
A	30962	Kitakoma-gun, Yamanashi, Japan
A	30963	Kitakoma-gun, Yamanashi, Japan
A	30976	Kiso-gun, Nagano, Japan
A	30977	Ohno-gun, Gifu, Japan
A	30985	Ohno-gun, Gifu, Japan
A	30964	Gdynia, Poland
A	31445	Surrey, England, UK
A	80048	Surrey, England, UK

(continued)

Table 1 (continued)

Haplotypes	Specimen	Geographic origin
A	N10	Magadan district, Magadan region, Russia
A	GAL14284	Denali National Park, Alaska, USA
A	GAL16735	Fairbanks, Alaska, USA
A	GAL5895	Serpentine Hot Springs, N. of Nome, Alaska, USA
A	GAL15453	North Pole, Alaska, USA
A	GAL15461	North Pole, Alaska, USA
A	GAL5505	Denali National Park, Alaska, USA
A	RET036-3	Zürich canton, Switzerland
A	RET309-3	Sogn og Fjordane, Norway
A	RET143-5	Kamchatka, Russia
A	RET141-2	Highlands & Islands Reg., Scotland, UK
A	N3	Ola district, Magadan region, Russia
A	N12	Ola district, Magadan region, Russia
A	N5	Magadan district, Magadan region, Russia
A	N13	Omsukchan district, Magadan region, Russia
A	N6	Magadan district, Magadan region, Russia
A	MP23	Bird Creek Campground, Anchorage, Alaska, USA
A	N15	Tenka district, Magadan region, Russia
A	MP24	Bird Creek Campground, Anchorage, Alaska, USA
A	N16	Anadyr district, Chukot Autonomous Region, Russia
A	N1	Ola district, Magadan region, Russia
A	N11	Ola district, Magadan region, Russia
A	GAL8950	Denali National Park, Alaska, USA
A	GAL5900	Serpentine Hot Springs, N. of Nome, Alaska, USA
A	GAL5946	Quartz Creek, E. of Nome, Alaska, USA
A	GAL16735-2	Fairbanks, Alaska, USA
A	GAL18071	Fairbanks, Alaska, USA
A	GAL16735-3	Fairbanks, Alaska, USA
A	GAL18076	Fairbanks, Alaska, USA
A	GAL15454	North Pole, Alaska, USA
A	GAL18134	Bonanza Creek LTER site, Alaska, USA
A	GAL18136	Bonanza Creek LTER site, Alaska, USA
A	GAL17899	Fairbanks, Alaska, USA
A	GAL18012-6	Fairbanks, Alaska, USA
A	GAL18012-2	Fairbanks, Alaska, USA
A	GAL18012-4	Fairbanks, Alaska, USA
A	GAL18810-2	Homer, Alaska, USA
A	GAL18810-1	Homer, Alaska, USA
A	GAL17691	Fairbanks, Alaska, USA
B	RET107-6	Highlands & Islands Reg., Scotland, UK
C	GAL2005	Bonanza Creek LTER site, Alaska, USA
D	RET264-7	Skamania Co., Washington, USA
D	GAL4247	Glacier Hwy, mile 27, Juneau, Alaska, USA
D	GAL4302	Glacier Hwy, mile 27, Juneau, Alaska, USA
D	RET136-2	Whatcom Co., Washington, USA

(continued)

Table 1 (continued)

Haplotypes	Specimen	Geographic origin
D	GAL3643	Glacier Hwy, mile 30, Juneau, Alaska, USA
E	RET149-1	Southern Highlands Prov., Tanzania
E	RET149-2	Southern Highlands Prov., Tanzania
F	N8	Magadan district, Magadan region, Russia
G	GAL18122	Bonanza Creek LTER site, Alaska, USA
H	GAL2814	Dalton Hwy, mile 122, Alaska, USA
I	GAL4810	Denali National Park, Alaska, USA
J	31452	Hampshire, England, UK
J	30965	Gdansk, Poland
J	RET152-6	Baden-Württemberg, Germany
J	RET152-8	Bavaria, Germany
K	30961	Aomori-shi, Aomori, Japan
L	30987	Queenstown, New Zealand
M	N4	Ola district, Magadan region, Russia
N	N14	Tenka district, Magadan region, Russia

that of Clade I discussed above. MDIV showed evidence for intermediate levels of gene flow ($M \sim 2.5$) between Alaskan and Eurasian populations and estimated no population divergence ($T \sim 0$) (Fig. 1b). Our analyses using MIGRATE suggested that migration between Alaska and Eurasia has been asymmetrically bidirectional with Alaska receiving more migrants ($M_{12} = 9,482.98$ and $M_{21} = 33.53$; M_{12} referring to the migration from Eurasia to Alaska, and M_{21} *vice versa*). Therefore, in simulations using Genetree, we assumed panmictic population structure. As expected, the coalescent-based genealogy did not show any historical population division (Fig. 2b). However, based on the fact that the specimens from the maritime rainforest ecoregion of the Pacific Northwest of North America formed a well-supported sister group (II/A) to the rest of Clade II in our previous multi-locus phylogenetic analyses (Geml et al. 2006, 2008), we tested in a separate analysis whether or not there was any gene flow between this group inhabiting a special habitat with a restricted distribution and the rest of Clade II, this latter including boreal Alaskan and Eurasian specimens. In this case, MDIV showed no migration ($M \sim 0$) and estimated a divergence time of $T \sim 1.5$ between the two populations, i.e. subclade II/A vs. Clade II (Fig. 1c). Nonetheless, the coalescent-based genealogy estimated under an assumption of population subdivision did not differ significantly from the one generated assuming panmixia (Fig. 2c).

4 Discussion

The main goal of this project was to estimate migration between Alaskan and non-Alaskan populations within distinct phylogenetic species in the *A. muscaria* species complex in order to test whether or not these fungi could have survived the

Table 2 Polymorphic sites in haplotypes collapsed after recoding indels and excluding infinite-sites violations from the original dataset for coalescent analyses. Position refers to that in the original alignment, site number is the designation of the given mutation as shown on Fig. 1, site type refers to transition (t), transversion (v), or deletion (-) change with regard to the consensus sequence. Character type indicates either parsimony-informative (i) or non-informative (-) sites. Capital letters refer to haplotype designations on Fig. 2

Phylogenetic species	Clade I	Clade II
Position	1111122223344455 16660136600132712668 00134960218778400483	1111111233456 2446672334558705061 366231200182485654280
Site number	11111111112 12345678901234567890	111111111122 123456789012345678901
Site Type	ttt-vt-ttttttttttt	ttttttttvtvvvtvvtt
Character Type	-i-i-ii-----i----	-----i--i-----
Haplotypes:		
A	C...	
B	T..2.....	G.....CT.....
C	.T...T.....	.G.....
D	.T.....C	..GC.CA.....T.
E	.T...T.C.....	...T..T...CC.....
F	.T...T...T...G..A..	C.....
G	..A...2.....T...	T.....
H	...2.....	A.....
I	...A.....	C.A.....
J	2.....T...	T.....
K	2.G..G.C.....	A.....
L	2.....	A.....
M	T.G.....	A.....
N	T.....	A.....
O		

LGM in Alaska. Because non-Alaskan populations most likely survived the LGM in refugia South of the major ice shields, the lack of migration between these and the Alaskan ones would suggest local survival of the latter, implying forest refugia in Alaska.

Overall, we found support for at least two endemic regional populations (haplotype D in Clade I, and haplotype D in Clade II) in different parts of Alaska, both of which exhibited genetic isolation and differentiation from other conspecific populations. Unfortunately, we could not estimate the times of divergence of the clades with certainty due to the lack of fossils and the great variance in nucleotide substitution rates in fungi. Nonetheless, it seems very likely that the major lineages (Clades I-III) separated well before the Pleistocene glacial cycles (Geml et al. 2006). On the other hand, the endemic regional populations likely represent groups that became isolated from the rest of their species in the Pleistocene and survived one or more glacial maxima in local refugia. Our findings support the existence of at least two independent such glacial forest refugia: (1) boreal forest in Interior Alaska; and

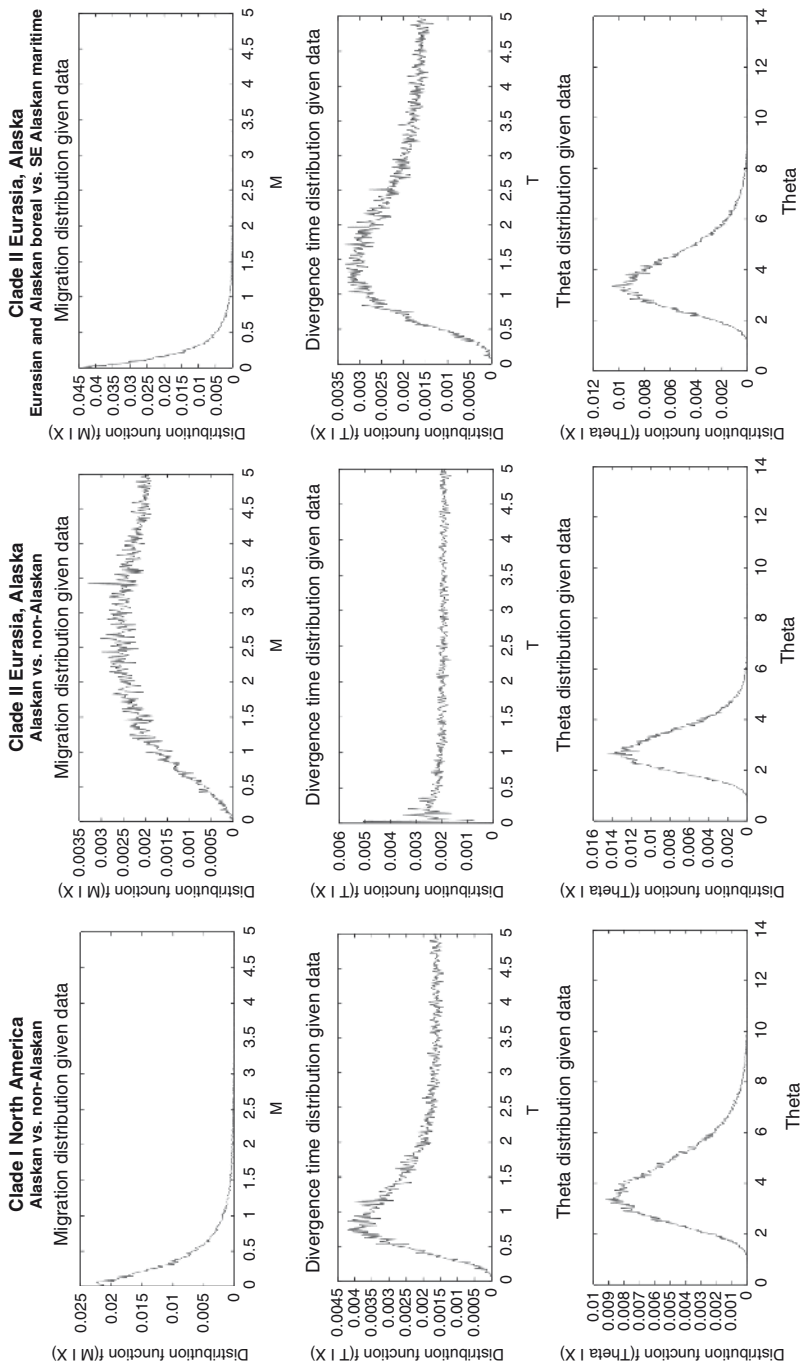


Fig. 1 Posterior probability distributions of migration (M), divergence time (T) and population mean mutation rate (Θ) between population pairs using MDIV. Estimates (axis X) with the highest distribution function (axis Y) values have the greatest probabilities. A and C show examples of zero migration and non-zero divergence time, while graphs in B indicate recent gene flow and no divergence between population pairs

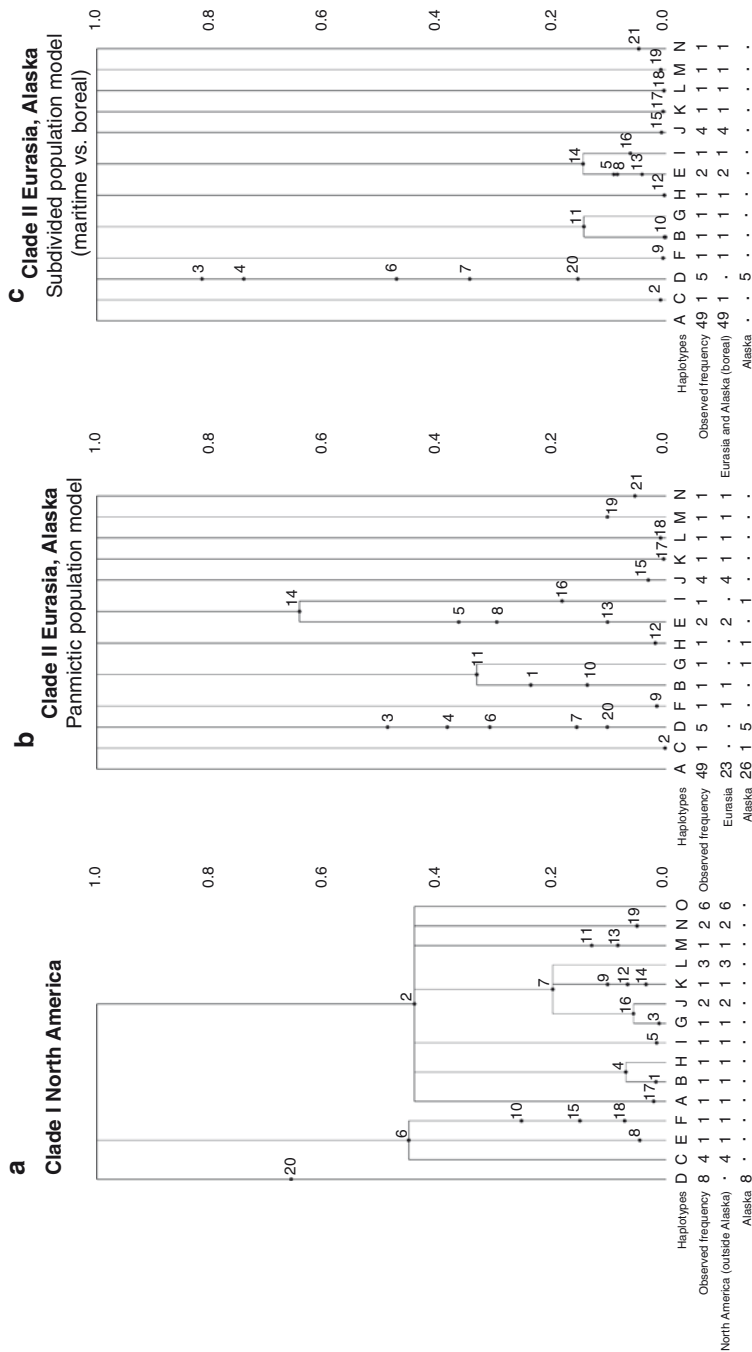


Fig. 2 Coalescent-based genealogies with the highest root probabilities showing the distribution of mutations. The inferred genealogies are based on 2 million simulations of the coalescent. The time scale is in coalescent units of $2N$, where N is the population size. Mutations and bifurcations are time ordered from the top (past) to the bottom (present). Mutation designations correspond to the site numbers in Table 2. The numbers below the tree designate the distinct haplotypes, their observed frequencies in total and in the different geographic regions

(2) maritime rainforest in Southeast Alaska and the Pacific Northwest (Fig. 3). Apart from this, many Alaskan haplotypes in Clade II were shared with specimens from Eurasia. Our results suggesting high migration, most likely both pre- and post-glacial, across the Bering Strait and the similar observed numbers of haplotypes endemic to either Eurasia or Alaska leave open the question whether boreal members of Clade II survived the LGM in Alaska or colonised Alaska from Asia in multiple times in postglacial events.

The possible existence of forest refugia in Interior and Southeast Alaska is also supported by several other independent lines of evidence. For example, although previous palynological biome reconstructions suggest that all of

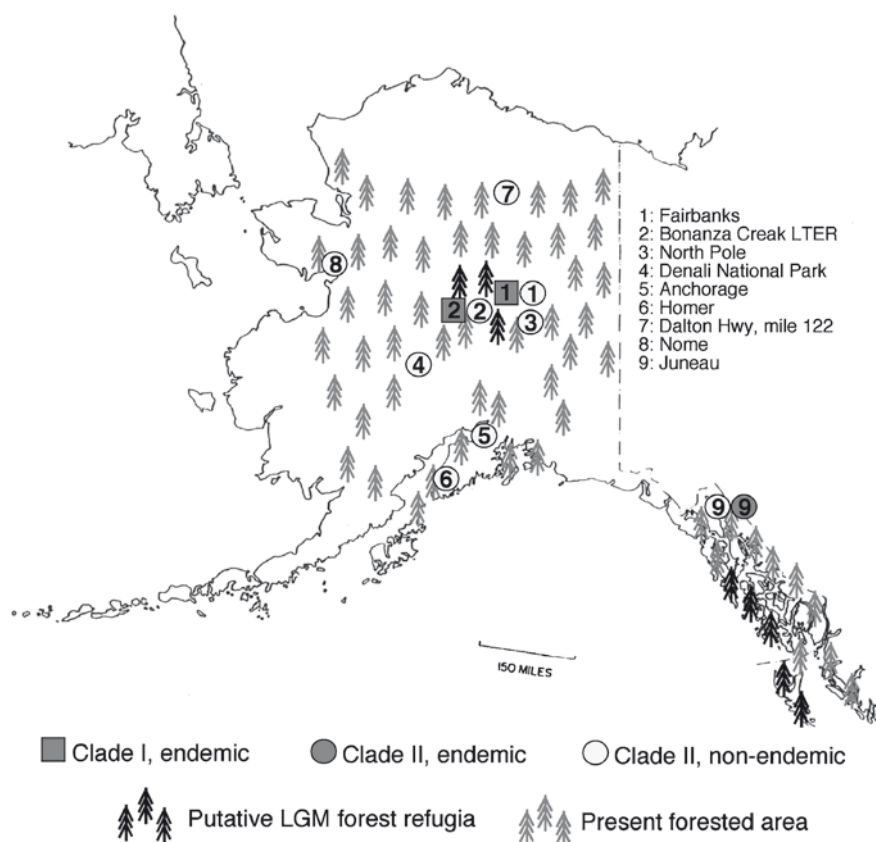


Fig. 3 Outline map of Alaska showing the collecting locations for the 43 Alaskan specimens included in this study. *Dark grey squares* and *circles* indicate sampling localities for endemic haplotypes, while *light grey circles* refer to haplotypes shared between Alaska and Eurasia. Black stylised spruce drawings mark putative forest refugia during the Last Glacial Maximum (LGM) suggested by this and other studies mentioned in the discussion. Grey tree figures indicate present forested area, without making a distinction between boreal and maritime forests of Interior and Southeast Alaska, respectively

Beringia was covered by arctic tundra (Edwards et al. 2000; Kaufman et al. 2004; Swanson 2003), recent pollen data by Brubaker et al. (2005) supports the theory that *Picea* forests were present in Eastern Beringia, at least in small fragments restricted to habitats with favourable microclimates. The Northernmost distribution of *Picea*, unlike that of *Betula* and *Populus*, is restricted, by definition, to the boreal region. Therefore, Brubaker's findings indicate the existence of the boreal forest biome in Alaska during the LGM. Similarly, Anderson et al. (2006), surveying forest stands across Northwestern North America, found several chloroplast DNA haplotypes of *Picea glauca* that were unique to Alaska, suggesting local survival. Maroja et al. (2007) analysed mitochondrial DNA sequence data from the spruce beetle (*Dendroctonus rufipennis*) and suggested that the postglacial range expansion of these beetles occurred from three refugia, one of which was in Interior Alaska.

Forest refugia in Southeast Alaska are also supported by genetic studies in several different taxa. For example, this is a region with high mammalian endemism due to its fragmented landscape and complex glacial history. Even animals, as mobile as wolves, have been shown to exhibit a strong signal of independent histories for the coastal and continental populations (Weckworth et al. 2005). Also, phylogenetic studies of Fleming and Cook (2002) on ermine (*Mustela erminea*) showed that there was at least one lineage strictly endemic to Southeast Alaska that likely survived locally during the Wisconsin glaciation. Locations for such glacial refugia in Southeast Alaska have been proposed by Carrarra et al. (2003) based on geological evidence.

In this paper, we demonstrated the use of ECM fungal taxa to indirectly infer glacial vegetation history. Therefore, our results are not only relevant to the mycological community, but to a broader biological community in general, particularly to those with a biogeographic interest.

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HALLUCINOGENIC SPECIES IN AMANITA MUSCARIA. DETERMINATION OF MUSCIMOL AND IBOTENIC ACID BY ION-INTERACTION HPLC

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ABSTRACT

Ibotenic acid and muscimol are considered the principal components responsible of the hallucinogenic properties of *Amanita muscaria*. In this paper a sensitive ion-interaction HPLC method is presented which permits the simultaneous determination of ibotenic acid and muscimol. A C₁₈ reverse phase is the stationary phase and the mobile phase is an aqueous solution of 5.0 mM octylammonium o-phosphate. A spectrophotometric comparative detection performed at 230 and 254 nm is employed. Detection limits are of 18 µg/L for ibotenic acid and of 30 µg/L for muscimol.

Different sample pretreatment processes were compared. The highest recovery yields together with the minimum matrix effect were obtained by a procedure which only consists in the sonication of the fresh sample and its squeezing. The extract obtained is then diluted as required and filtered (0.2 µm) before injection. This preparation offers the further advantage to maximally preserve the native composition.

The method was applied in the analysis of *Amanita muscaria* and permitted to evaluate the content of muscimol and ibotenic acid in heads and stems of the mushrooms. The same analysis performed for samples of *Amanita citrina* confirmed the absence in this species of both ibotenic acid and muscimol.

INTRODUCTION

Amanita muscaria is generally more known for its toxic properties than for the psychotropic ones and the literature methods concerning the analysis of *Amanita* mostly regard identification and determination of its toxic components, such as muscarine, amatoxins and phallotoxins.¹⁻⁵

It is on the other hand reported^{6,7} that in North Europe countries, and especially in Siberia, *Amanita muscaria* hallucinogenic properties were known since 4.000 b.C. The "Rig-Veda", which is considered the first politeist text, mentions *Amanita* as the "God plant" because of its exceptional properties to transmit prodigious powers.⁸ The species responsible for the psychotropic effects, which have been now identified are: ibotenic acid (amino-(3-hydroxy-5-isoxazolyl)acetic acid) and its decarboxylation product muscimol (3-hydroxy-5-aminomethylisoxazole).⁸⁻¹⁰ Their structures are presented in Fig. 1.

These compounds are receiving great interest in psychiatric medicine because of their potential ability to be employed as an alternative to synthetic drugs or to be used as possible models for the development and preparation of new drugs devoted to the treatment of psycho-diseases.⁹ Analytical methods are therefore required for extraction, purification and determination of these compounds in *Amanita* mushrooms.

Only two determination methods are reported in literature. One makes use of a microbore column and UV detection after reaction with ninhydrin in presence of potassium cyanide.⁹ The other (direct source¹⁰ is not easily available) is a HPLC method, which makes use of an amino-bonded stationary phase: the preparation of the sample requires two SPE separation processes respectively performed with Dowex 1-X8 resin (acetate form) for the extraction of ibotenic acid and with alumina-silica gel for muscimol. Detection limits are respectively 18 and 10 mg/L of ibotenic acid and muscimol.

This paper presents a sensitive HPLC ion-interaction method for the simultaneous separation and determination of muscimol and ibotenic acid in the extract which can be easily obtained by squeezing the fresh sample of

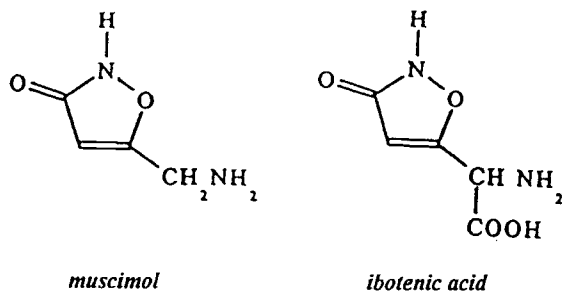


Figure 1. Chemical structures of the analytes.

Amanita muscaria. No other pretreatment is required (a part a suitable dilution and 0.22 μ m filtration) and this feature is of particular importance because the native composition is, as far as possible, preserved and conditions for the taking place of decarboxylation reactions (which transform ibotenic acid into muscimol) are prevented. Measurements are also performed for samples of *Amanita citrina*, which is reported not to contain psychotropic compounds.⁸

EXPERIMENTAL

Chemicals and Reagents

Ultrapure water from Millipore MilliQ (Milford, MA) system was used for the preparation of solutions. Ibotenic acid and muscimol are Sigma Chemical Co. reagents. Pentylamine, hexylamine, heptylamine, octylamine, nonylamine, ortho-phosphoric acid and HPLC-grade acetonitrile were Fluka (Buchs, Switzerland) chemicals. All other reagents were C.Erba (Milano, Italy).

Apparatus

The chromatographic analyses were performed with a Merck-Hitachi (Tokyo, Japan) Lichrograph chromatograph Model L- 6200 equipped with a two channel D-2500 chromato-integrator interfaced with a UV-Vis detector L-4200.

The absorbance spectra A/λ were recorded by a Hitachi (Tokyo, Japan) mod. 150-20 spectrophotometer. The analyses were performed at two different wavelengths, namely 230 nm, which corresponds to the maximum absorbance for the two analytes, and 254 nm, at which absorptivity coefficient for ibotenic acid is much lower than at 230 nm. The choice of the latter wavelength was due to: a) a lower matrix interference effect as concerns muscimol determination and b) to confirm, under different conditions, the analyte identification and determination.

For pH measurement a Metrohm (Herisau, Switzerland) 654 pH-meter provided with a combined glass-calomel electrode was employed.

Chromatographic Conditions

A Phase Separations (Desidee, CLWYD, UK) Spherisorb S5 ODS-2 (5 μm , 250 x 4.6 mm) cartridge was used together with a guard pre-column Merck (Darmstadt, Germany) Lichrospher RP-18 (5 μm).

The mobile phases were aqueous solutions of the ion-interaction reagents. The use of orthophosphate and salicylate of different alkylamine (namely pentylamine, hexylamine, heptylamine, nonylamine and decylamine) at different pH values (3.0, 6.4 and 8.0) was compared. The solutions were prepared by adding to the amount of the amine (weighted to prepare the desired molar concentration), o-phosphoric acid or salicylic acid up to the desired pH value.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 hour was necessary at flow-rate of 1.0 mL/min. When in use, overnight the flow-rate of the mobile phase was maintained at 0.1 mL/min. After use or anyway after three days of use, the column was washed and a new mobile phase freshly prepared.

The washing steps were performed with water (1.0 mL/min for 30 min), a 50/50 v/v water/acetonitrile mixture (1.0 mL/min for one hour), and finally acetonitrile (0.7 mL/min for 20 min). This procedure is necessary, especially when working with real matrices, to remove highly retained lipophilic matter which doesn't elute in aqueous mobile phase, but could affect column modification. Retention intra-day repeatability was always within 3% for the same eluent preparation and inter-day reproducibility for different preparations was always within 6%.

Our results fit the model according to which the ion-interaction reagent contained in the mobile phase is bound onto the surface of the stationary phase through adsorption and electrostatic forces, giving rise to an electrical double layer. The interaction properties of the original reverse phase packing material are therefore modified and the modified surface is able to simultaneously retain cationic and anionic species.¹¹⁻¹⁴

Sample Preparation

The mushrooms investigated were collected in October in Piedmont (North Italy) and analyzed just within three days after sampling. Alternatively the mushrooms can be frozen soon after picking and cleaning and preserved until the analysis, when they are defrosted and treated as described for the fresh ones.

Different extraction procedures were experimented and compared. The aim was to obtain the maximum recovery yield, taking into account that conditions for the decarboxilation reactions to take place (which transform ibotenic acid into muscimol) must be avoided as much as possible.

The extraction procedures experimented were:

- a) solid phase extraction (SPE) employing an anionic-exchange column (QMA Waters) for ibotenic acid and a SPE-C18 for muscimol.
- b) liquid-liquid extraction, employing and comparing different extracting solvents mixtures, namely ethanol-water, methanol-water, ethanol-methanol-water in different volume ratios.
- c) liquid-liquid extraction, after removal of fats by Soxhlet extractions, respectively performed with chloroform, diethylether and hexane.
- d) pounding, sonication and water extraction.

Solid phase extraction (method a) and liquid extraction (method b) led to very low selectivity for the studied analytes and to a consequently heavy matrix effect in the chromatographic analysis. Soxhlet fat extraction also led to a partial extraction of the analytes. The procedure d) was therefore applied for the pretreatment of the sample, also taking into account that the conditions at minimum affect the speciation and at maximum avoid the contamination of the sample. The procedure is described in detail, as follows.

Procedure Followed

The mushrooms were cleaned and the heads and stems were separately pounded in a mortar. After 15 min of sonication treatment, the juice was separated from the pulp and resulted to be about 90% of the fresh weight. In order to improve the recovery of the analytes the pulp was washed twice with ultrapure water. The juice was diluted with ultrapure water and filtered through a 0.22 μm nylon membrane before the injection in the HPLC apparatus. The optimum final dilution prior to the HPLC analysis was 1/1300 v/v.

RESULTS AND DISCUSSION

As mentioned, no example can be found in literature of the simultaneous separation of muscimol and ibotenic acid.

By considering the molecular structure of the analytes (see Fig.1) and their different hydrophilicities, the ion-interaction chromatography seemed to be particularly suitable for their separation.

On the basis of previous results,¹¹⁻¹³ it was pointed out that the good applicability of the method greatly depends on its versatility and the dependance of retention on many variables which must be optimized. Our aim was the development of a method which offers the maximum sensitivity for the analytes, together with the minimum interference effect by the several other components of the matrix.

Method Development

The ion-interaction chromatographic method was optimized as concerns the pH of the mobile phase and the ion-interaction reagent chemical properties. The use of different pH values of the mobile phase showed no significant influence, as concerns both retention and sensitivity, in the pH range between 3.0 and 8.0: a pH value of 6.4 was chosen as more similar to the natural tissue.

As concerns the choice of the ion-interaction reagent, the comparison of salicylate and o-phosphate as counter anions showed that better sensitivities could be gained with o-phosphate. The use of alkylammonium o-phosphate

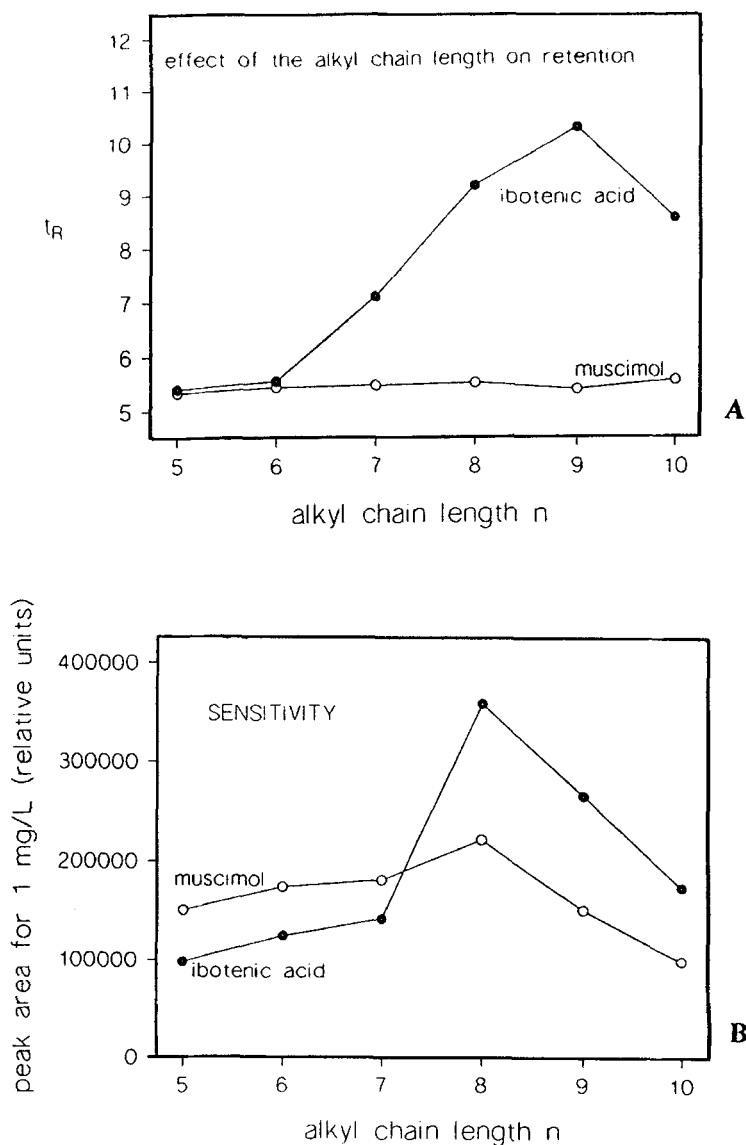


Figure 2. Plot of resolution (A) and of sensitivity (B) vs carbon chain length of the ion interaction reagent (aqueous 5.0×10^{-3} mol/L alkylammonium orthophosphate, pH 6.4) for the two hallucinogenic compounds.

salts with the alkyl chain length n varying between 5 (pentylamine) and 10 (decylamine) was then compared. Fig.2A shows that the effect of n on retention is very relevant for ibotenic acid, in agreement with its more hydrophilic properties, while it can be considered as negligible for muscimol.

As a consequence, also the resolution between the two analytes is greatly affected by the alkyl chain length. Resolution R_s shows its maximum value for $n=9$ ($R_s=3.04$) and is also reasonably high for $n=8$ ($R_s=2.28$).

On the other hand, previous studies¹⁴ have shown that the alkyl chain length of the ion-interaction reagent can also affect analyte sensitivity. The behaviour is also followed by the analytes investigated here (Fig.2B): for both analytes the best sensitivity is achieved for $n=8$. These results suggested the use, as mobile phase, of a 5.0 mM octylammonium o-phosphate aqueous solution at pH=6.4.

As concerns UV detection, this was performed both at 230 and 254 nm, at which wavelengths the two analytes show significantly different absorptivity values: the comparative detection at two different wavelengths can be helpfully employed to confirm the identification and the quantitation or to better overcome matrix interference effects.

Fig.3 shows a typical separation obtained under the optimized chromatographic conditions, with detection at $\lambda=230$ nm, of a mixture of muscimol (0.150 mg/L) and ibotenic acid (0.100 mg/L).

The calibration curves built for ibotenic acid in the concentration range between 0.05 and 1.00 mg/L, and for muscimol in the concentration range between 0.10 and 3.00 mg/L, showed a good correlation between peak area and concentration: the plots could be fitted by straight lines with correlation coefficients always higher than 0.995. From the sensitivity data and for a signal/ noise ratio = 3, detection levels as low as 30 $\mu\text{g/L}$ and 18 $\mu\text{g/L}$ were respectively evaluated for muscimol and ibotenic acid.

***Amanita Muscaria* and *Amanita Citrina* Analysis**

The method was then applied on the analysis of the two extracts respectively obtained, as described in the experimental section, from heads and stems of *Amanita muscaria* and *Amanita citrina*.

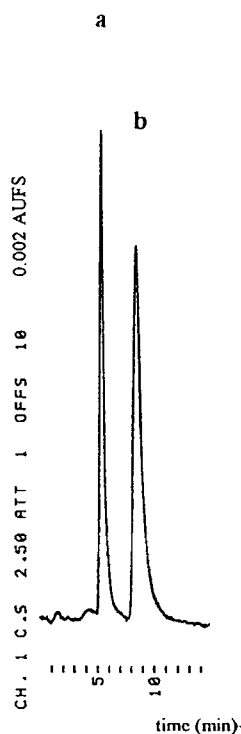


Figure 3. Chromatogram of the standard solution containing 0.150 mg/L of muscimol (a) and 0.100 mg/L of ibotenic acid (b). Experimental conditions. Phase Separation S5 ODS-2 250x4.6 mm column. Mobile phase: aqueous 5.0×10^{-3} mol/L octylammonium α -phosphate, pH 6.4. Flow-rate: 1.0 mL/min. Spectrophotometric detection at 230 nm.

The ion-interaction chromatographic method developed here shows advantages of selectivity towards more lipophilic components present in the matrix. This is likely due to the characteristics of the method which utilizes an aqueous mobile phase and is based on the capability of the analytes to form ion-pairs.

As expected, in agreement with botanic information⁸ *Amanita citrina* did not show the presence, at least at the detection limit, of both ibotenic acid and muscimol.

As concerns *Amanita muscaria*, Fig. 4 shows, as a comparison, typical chromatograms respectively recorded at 230 nm and 254 nm for the head extract. In agreement with molar absorptivity values, sensitivity for muscimol

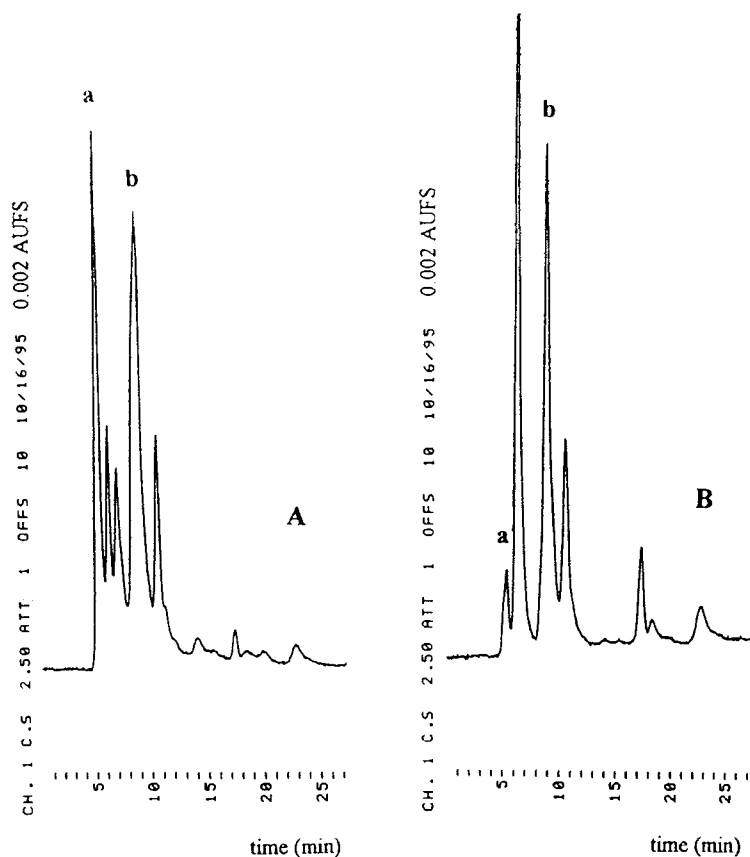


Figure 4. Chromatogram of the diluted (1/1300 w/w) juice from *A. muscaria* heads at 230 (A) and 254 nm (B). Experimental conditions as in Figure 3. a: muscimol; b: ibotenic acid.

is much lower at 254 than at 230 nm, but the interference-freedom is much higher. No significant difference can instead be observed at the two wavelengths for ibotenic acid.

Quantitation of the two analytes was performed by a standard addition method taking into consideration the chromatographic response in terms of both peak area and peak height at the two wavelengths. On average, the quantitative results always agree within 15%. As expected, the amount found

for the two compounds is always higher in heads than in stems. The mean values, referred to fresh mushroom, was found to be: for muscimol 0.38 ± 0.03 g/Kg in heads and 0.08 ± 0.01 g/Kg in stems; for ibotenic acid 0.99 ± 0.01 g/Kg in heads and 0.23 ± 0.03 g/Kg in stems.

The only available literature data⁹ report for heads of fresh mushroom amounts of respectively 41.8 mg/Kg of muscimol and 86.6 mg/Kg of ibotenic acid.

The higher content we found might be ascribed, besides the different geographical origin of the samples, to the treatment processes performed prior to the analysis, which in the method here proposed at maximum preserves native composition. No particular pretreatment, extraction process or derivatization reaction is in fact required, a part from the squeezing of the mushroom and the subsequent dilution and filtration of the extract. This extraction process and the absence of contaminating reagents can be of practical interest for subsequent use of the extract.

ACKNOWLEDGMENT

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Amanita muscaria toxicosis in two dogs

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Abstract

Objective: To report the manifestations, history, and pathophysiologic basis of disease in 2 dogs with *Amanita muscaria* toxicosis.

Case summaries: Two dogs were evaluated for an acute onset of gastroenteritis and seizures. *A. muscaria* toxicosis was suspected in each dog after confirmation of environmental exposure and visualization of ingested mushrooms in vomitus. The diagnosis was confirmed following identification of toxic *Amanita* metabolites in the urine and serum of each dog. Administration of supportive and symptomatic therapies resulted in the complete recovery of each animal.

Unique information provided: Ingestion of the mushroom, *A. muscaria*, by dogs can result in acute gastrointestinal distress that precedes a potentially life-threatening central neurologic syndrome characterized by seizures, tremors, and somnolence. Central nervous system dysfunction results primarily from the actions of ibotenic acid and its decarboxylation product, muscimol, which are analogues of the neurotransmitters glutamate and γ -aminobutyric acid (GABA), respectively. Identification of these toxins in the urine and serum of affected dogs using high-performance liquid chromatography (HPLC) provides a definitive diagnosis.

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Keywords: fly agaric, HPLC, ibotenic acid, muscimol, mycology

Introduction

Several species of *Amanita* mushrooms have been previously associated with clinically significant human and animal toxicoses.^{1–6} Veterinary publications concerning toxicoses associated with *Amanita* species are rare.^{2–5} Reports of canine ingestion of *A. ocreata* and *A. phalloides*, which contain cyclopeptide amatoxins, describe clinical signs of gastroenteritis and fulminant hepatic necrosis.^{1,2,4}

Amanita muscaria, commonly referred to as the fly agaric, and *A. pantherina* mushrooms are the 2 species most frequently implicated in the pantherina–muscaria poisoning syndrome in humans.^{6,7} This syndrome is primarily characterized by signs of central nervous system (CNS) dysfunction including hallucinations, auditory and visual hypersensitivity, tremors, convulsions, and somnolence. Clinical manifestations result

from the effects of 2 main toxins, ibotenic acid and muscimol, which interfere with normal glutamate (Glu) and γ -aminobutyric acid (GABA)-mediated neurotransmission.⁷ In humans, accidental ingestion of *A. pantherina* is the most common reason for toxicosis associated with this species. However, the psychoactive properties of *A. muscaria* have been recognized for centuries, and voluntary ingestion of fly agaric for both ritualistic and recreational purposes result in most human intoxications requiring medical treatment.^{7–9}

Presumptive cases of *A. pantherina* poisoning have been reported to cause muscle tremors, somnolence, delirium, and convulsions in cats and dogs,^{3,5} but to the authors' knowledge, descriptions of disease resulting from ingestion of *A. muscaria* have not appeared previously in the veterinary literature. The purpose of this case series is to describe the clinical signs, history, and pathophysiologic basis of disease in 2 dogs with *A. muscaria* poisoning.

Case Summaries

Case 1

A 2-year-old, intact male, English setter (Dog 1) was referred for evaluation of cluster seizures and diarrhea.

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The dog was reportedly normal the previous evening, but was discovered the next morning by the owner in its outdoor enclosure in lateral recumbency and covered in diarrheic feces. Then, while transporting the dog to the referring veterinarian, the owner witnessed the first of 3 generalized, tonic-clonic seizures. Upon arrival, the referring veterinarian reported that the dog was hypersalivating, stuporous, and had profuse small bowel diarrhea. Results of a packed cell volume, total protein, and blood glucose concentration were within reference ranges. No abnormalities were found on fecal flotation. The veterinarian also observed a partial facial seizure that subsequently became generalized. Intravenous (IV) diazepam^a (0.23 mg/kg) temporarily abated the seizure. Approximately 20 minutes later, a second generalized tonic-clonic seizure occurred and a second dose of diazepam^a (0.23 mg/kg IV) was administered. Over the course of the next 2 hours, the dog was reported to have 2 additional episodes of diarrhea and several, intermittent bouts of facial twitching, of which 2 progressed to generalized seizure activity. A third, identical IV dose of diazepam^a was administered and the dog was referred for further diagnosis and treatment.

At the time of presentation, the dog was in status epilepticus. Additional physical examination abnormalities recorded at the time of admission included miotic pupils, fluid distended bowel loops, and a cardiac bradyarrhythmia associated with occasional peripheral pulse deficits. An IV bolus of diazepam^a (0.48 mg/kg) was administered and seizure activity ceased. Metabolic (i.e., hypoglycemia, hepatic insufficiency, hypoadrenocorticism), toxic (i.e., chlorinated hydrocarbons, lead, tremorigenic mycotoxins, organophosphates/carbamates, *Amanita* spp. mushrooms), and infectious diseases (i.e., canine distemper) were considered as the primary differential diagnoses for the clinical signs noted in this dog. The SpO₂ was 97% in room air, and an electrocardiogram (ECG) identified a high-grade, second-degree atrioventricular block, with the ratio of atrial and ventricular complexes being fixed at 3:1. A complete blood count (CBC) was consistent with a stress leukogram. Metabolic causes of disease were considered unlikely after results of a serum biochemical profile, urinalysis, activated partial thromboplastin time, and prothrombin time were all within reference ranges. The blood lead concentration in this dog was less than the detectable limit of the assay (<50 ppb). Atropine^b (0.05 mg/kg IV and 0.15 mg/kg SQ) was subsequently administered and abolished the atrioventricular block. The dog was then bathed with warm, soapy water to remove fecal soiling before the placement of an additional IV catheter. Crystalloid therapy^c with supplemental potassium chloride

(20 mEq/L) was administered at a rate of 4 mL/kg/h to maintain hydration and account for ongoing gastrointestinal fluid loss.

While being instrumented for continuous ECG telemetry and pulse oximetry, the dog appeared delirious and manifested several episodes of fly-biting behavioral stereotypy that preceded another generalized tonic-clonic seizure. The seizure activity abated after the administration of 2 IV doses of diazepam^a (0.48 mg/kg, followed immediately by 0.96 mg/kg). A loading dose of phenobarbital^d (8.6 mg/kg) was then given as a slow IV infusion, during which time the dog had 2 cluster seizures that prompted the initiation of a diazepam^a constant rate infusion (CRI) at a dosage of 1 mg/kg/h. Additionally, phenobarbital^d maintenance therapy (2.2 mg/kg q 12 h) was initiated intramuscularly (IM). The dog was maintained on the diazepam^a CRI for approximately 2 hours, after which time, 2 additional cluster seizures were observed that failed to completely respond to 2 IV boluses of diazepam (0.96 mg/kg). Propofol^e was then administered (3 mg/kg IV loading bolus, followed by 0.1 mg/kg/min IV CRI) which resulted in cessation of the seizures. After 6 hours of the propofol^e CRI, attempts were made to wean the propofol^e therapy. However, as soon as the infusion was discontinued, appendicular and facial muscular tremors were observed, and the propofol^e CRI was restarted at the same dosage.

Approximately 2 hours later, the dog vomited twice in rapid succession while being maintained on the propofol^e infusion. The vomitus contained partially digested commercial dog kibble and multiple fragments of organic material that resembled mushroom caps and stems. Oropharyngeal and esophageal suction was performed to remove residual vomitus. Venous blood gas analysis was within reference ranges.

The dog experienced an additional generalized seizure 1 hour later. An electroencephalogram^f (EEG) was obtained that revealed intermittent bursts of epileptiform activity that were associated with clinically apparent facial motor disturbances. The propofol^e CRI dosage was incrementally escalated to 0.25 mg/kg/min in an attempt to achieve burst suppression on the EEG, but continual interpretation of the EEG was complicated by a large amount of motion artifact associated with the frequently occurring facial muscular tremors and fasciculations. Therapy with methocarbamol^g (50 mg/kg IV q 8 h) was also initiated, which completely abolished the appendicular muscular tremors and markedly reduced the frequency and severity of the facial tremors.

Over the next 6 hours, the muscular tremors largely abated and the dog was gradually weaned from the propofol^e infusion. The dog remained recumbent and

somnolent for an additional 10 hours. Thoracic radiographs were obtained to examine for evidence of aspiration pneumonia; no abnormalities were observed. Approximately 40 hours after admission, the dog was able to assume a sternal position and was eating and drinking voluntarily, so was weaned from IV fluid therapy. The dog was switched from IM to oral maintenance phenobarbital^h therapy (2.9 mg/kg PO q 12 h). By the third day of hospitalization, the dog appeared clinically normal. The methocarbamol^g was discontinued and the dog was discharged to the owner with instructions to continue the phenobarbital^h therapy as prescribed.

The owners of the dog were questioned about possible environmental exposure to mushrooms, and subsequently, they provided intact and partially eaten mushrooms found in the dog's living area. Samples of partially eaten mushrooms provided by the owner were submitted to a botanist and veterinary toxicologist and confirmed to be *A. muscaria* var. *formosa* (Figure 1).

Materials and Methods for High-Performance Liquid Chromatographic (HPLC) Identification of Ibotenic Acid and Muscimol

The HPLC procedure utilized was a modification of the ion-interaction method previously described by Genaro et al.¹⁰ Briefly, the following chemical grade reagents were obtained from commercial sources: ibotenic acid,ⁱ muscimol,^j octylamine,^k phosphoric acid,^l methylene chloride,^m and methanol.ⁿ

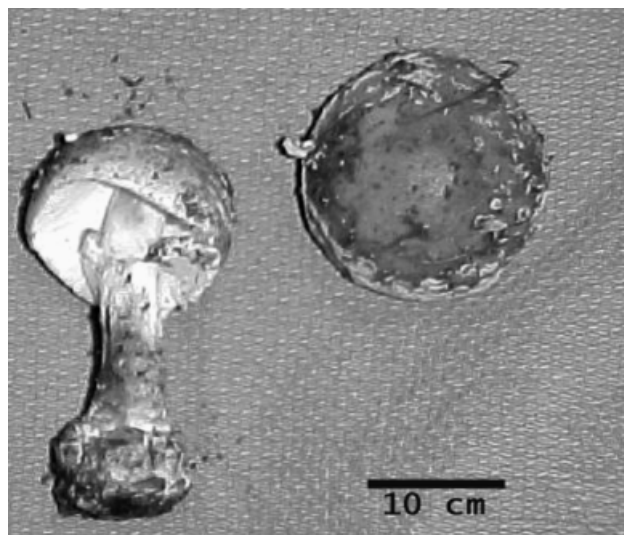


Figure 1: Photograph of *Amanita muscaria* var. *formosa*. Intact mushroom (left), and orange-yellow pigmented cap with white 'warts' (right) that is characteristic of mature mushrooms inhabiting the eastern United States.

Sample preparation of intact mushrooms involved cleansing of a 10 g mixed sample of previously identified *A. muscaria* stems and caps with ultrapure water,^o crushing the sample in a mortar, followed by extraction with 20 mL of methanol.ⁿ The resulting solution was filtered through a 0.22 μ m micropore nylon membrane before injection into the HPLC apparatus. To prepare urine and serum samples, 1 mL aliquots were mixed with 3 mL of a 90%/10% vol/vol mixture of methylene chloride^m/methanol,ⁿ agitated for 10 minutes, and allowed to settle. The bottom layer was removed, evaporated,^p and subsequently reconstituted with 100 μ L of methanolⁿ for injection into the HPLC apparatus.

The chromatographic apparatus utilized was a commercial HPLC system^q interfaced with proprietary analytical software.^r A 7 μ m, 250 $\times$ 4.6 mm reverse phase column^s was used together with a guard pre-column.^t

The mobile phase was created by adding phosphoric acid to a 5 mM aqueous solution of octylamine,^k titrated to a pH of 6.4. The chromatographic system was conditioned until a stable baseline signal was obtained at a flow rate of 1.0 mL/min. Spectrophotometric detection was performed at 230 nm. Reference standards were made of ibotenic acid and muscimol. Lower detection limits of the HPLC assay were 0.006 μ g/mL for ibotenic acid and 0.024 μ g/mL for muscimol.

Urine and serum ibotenic acid concentrations determined by HPLC were 0.706 and 0.239 μ g/mL, respectively, in Dog 1. Muscimol was also present in the serum and urine of Dog 1 at concentrations of 0.083 and 4.814 μ g/mL, respectively. Serum and urine samples obtained from a healthy control dog were simultaneously assayed using HPLC for ibotenic acid and muscimol. Both substances were undetectable in test samples from the control dog. HPLC analysis of an extract prepared from partially eaten mushrooms from Dog 1 yielded concentrations of ibotenic acid and muscimol that exceeded the upper detection limits of the assay.

Two months following discharge, during follow-up telephone conversations with the owner and referring veterinarian, Dog 1 was reported to be clinically normal with no additional seizures or gastrointestinal signs observed. At the 2-month telephone contact, the serum phenobarbital concentration was reportedly 19.3 μ g/mL (therapeutic range 15–40 μ g/mL). Dog 1 is currently being weaned off of phenobarbital^h therapy.

Case 2

A 4-year-old, castrated male, mixed-breed dog (Dog 2) was evaluated for somnolence, ptyalism, vomiting, and diarrhea of 12 hours duration. Upon examination, the dog was approximately 6% dehydrated, hypersalivating, and had miotic pupils; other physical parameters

were normal. A CBC, serum biochemical profile, and urinalysis were performed, and the dog was admitted for IV fluid therapy and continued observation. The results of the CBC and urinalysis were within reference ranges. Abnormalities present on the serum biochemical profile included an elevated alanine aminotransferase (ALT) (139 U/L; reference range 17–66 U/L) and hyperglycemia (130 mg/dL; reference range 84–118 mg/dL). During placement of a peripheral IV catheter, the dog was observed to have a generalized tonic-clonic seizure that abated following the administration of diazepam^a (0.55 mg/kg IV). A crystalloid solution^c was administered at a rate of 14 mL/kg/h for the subsequent 6 hours to replace the hydration deficit, after which time the fluid rate was decreased to 2.3 mL/kg/h.

The ptialism gradually subsided within 12 hours of admission, although the dog remained somnolent throughout this period. Approximately 18 hours after presentation, a second generalized tonic-clonic seizure, that lasted approximately for 1 minute, was witnessed. An IV bolus of phenobarbital^d (6.5 mg/kg) was administered, and maintenance therapy with phenobarbital^h (2.1 mg/kg PO q 12 h) was initiated. The dog appeared clinically normal approximately 12 hours later and was discharged with instructions to continue the phenobarbital^h therapy. The owners visually confirmed the presence of *A. muscaria* mushrooms growing in close proximity to the dog's outdoor enclosure. Serum concentrations of ibotenic acid and muscimol, determined using the HPLC method described for Dog 1, were 0.858 and 0.305 µg/mL, respectively. The urine concentration of muscimol was 6.909 µg/mL. The urine concentration of ibotenic acid was below the detection limit of the HPLC assay in Dog 2. The owner of Dog 2 was contacted for approximately 3 weeks after discharge and reported that the dog appeared clinically normal.

Discussion

The cases presented here demonstrate that *A. muscaria* toxicosis has the potential to cause an acute and severe clinical syndrome in dogs characterized by gastroenteritis and CNS dysfunction from which rapid and complete recovery can be expected with supportive and symptomatic therapies. *A. muscaria* mushrooms are common inhabitants of temperate coniferous and deciduous forests of many continents of the world, including North and South America, Asia, and Europe. There are 2 varieties of *A. muscaria* typically found in the United States. The characteristic brilliant scarlet red pileus with accompanying white surface 'warts' usually facilitates visual identification of mature *A. muscaria* var. *muscaria* that is found in the western United States. *A. muscaria* var. *formosa* is the eastern North American

variety and has a cap with a more yellow-orange hue. The growth season of *A. muscaria* typically lasts from early summer to late autumn.

A. muscaria was originally named following the isolation of muscarine from the mushroom and it was initially speculated that the clinical poisoning resulted from the toxic effects of muscarine. However, muscarine was later discredited as the primary toxin responsible for the clinical manifestations of fly agaric poisoning in humans.^{6–12} The concentrations of muscarine found in *A. muscaria*, although variable, are generally considered insufficient to cause the pantherina-muscaria syndrome, and explain the paucity of cholinergic clinical signs demonstrated by humans with *A. muscaria* toxicosis.^{9,13} The quaternary ammonium group present on muscarine prohibits entrance into the CNS, and thus muscarine functions as a competitive muscarinic receptor agonist in the peripheral nervous system, and is not subject to acetylcholinesterase-mediated degradation.¹¹ Muscarinic cholinergic receptors capable of binding muscarine are found in highest concentrations in the cardiac muscle and nodes, smooth muscle, and glands.⁹ Both dogs reported here displayed variably severe degrees of cholinergic signs, such as ptialism, miosis, vomiting, diarrhea, and second degree atrioventricular block. Cholinergic crises are rarely reported in adult humans with pantherina-muscaria poisoning which results from recreational or ritualistic consumption of fly agaric. Occasionally, however, nausea and vomiting have been observed in children with *A. muscaria* toxicosis that results from the ingestion of large quantities of mushrooms, as was suspected in both the dogs described here.^{7,13} Experimental peritoneal injections of *A. muscaria* extracts into rodents have been shown to decrease acetylcholinesterase activity.⁷ It is also possible that the gastrointestinal manifestations that accompany some cases of pantherina-muscaria poisoning are caused by an unidentified toxin.

The CNS derangements that are characteristic of pantherina-muscaria poisoning in humans and animals can be primarily attributed to the chemically related toxic isoxazole amino acid metabolites, ibotenic acid, and muscimol.^{7,10–12} Ibotenic acid is synthesized by the mushroom, whereas muscimol is formed by decarboxylation of ibotenic acid.^{7,11} The highest concentrations of toxic metabolites are found within the pileus of the mushroom, especially in the flesh immediately beneath the external cuticle. Both amino acids have been shown to be qualitatively present in *A. muscaria* species regardless of the geographic origin of the mushroom, but toxin content may quantitatively vary with both geographic site of origin, season harvested and, in instances of voluntary consumption, the method of

preparation.^{7,12} In addition, it has been previously reported that *A. muscaria* mushrooms consumed in September are more often associated with nausea and vomiting and have less potent hallucinogenic effects than those ingested earlier in the summer.¹⁵ In the dogs described here, intoxications occurred in late September and early October.

Both ibotenic acid and muscimol, although zwitterionic structures, are capable of crossing the blood–brain barrier by active transport, which explains their ability to produce CNS dysfunction.⁹ Ibotenic acid and muscimol are alkaloids that biochemically function *in vivo* as agonists of the neurotransmitters Glu and GABA, respectively.^{10–12} The amino acid Glu and its conformational analogs, which include ibotenic acid, mediate most of the excitatory synaptic neurotransmission in the mammalian brain through 2 distinct classes of Glu receptors, ligand-gated ionotropic receptors (iGlu) and G protein-coupled metaboreceptors (mGlu). Experimentally, ibotenic acid pharmacologically activates the *N*-methyl-D-aspartate subclass of iGlu, and acts as an agonist of variable potency at several distinct mGlu subfamilies.¹⁶ Its interaction with these Glu receptors is responsible for the signs of CNS excitation, including seizures, muscle tremors, and hallucinations that are the clinical hallmarks of acute *A. muscaria* toxicosis.¹⁶ Intraparenchymal delivery of ibotenic acid into rodent hippocampi has also been previously used as a clinical and electroencephalographic excitotoxin-induced seizure model.¹⁷ In most naturally occurring toxicoses associated with *A. muscaria* ingestion, the toxic effects of ibotenic acid are largely due to neuronal functional derangements, as morphologic brain damage is not a consistent feature of the pantherina–muscara poisoning syndrome. However, repeated local and systemic experimental administration of ibotenic acid to laboratory animals induces degenerative morphologic neuronal and glial lesions in basal nuclei.^{7,17}

Muscimol exerts a primarily depressant effect on the CNS which occurs through multiple interactions of the metabolite with GABA-mediated inhibitory neurotransmission. Muscimol is a potent and biologically active conformational analog of GABA, capable of *in vivo* activation of postsynaptic GABA receptors.^{7,11} Experimental *in vitro* studies in rodent models suggest that muscimol has a high affinity for both postsynaptic GABA receptors as well as autoregulatory receptors that may function in release of GABA from presynaptic neurons.¹⁰ Muscimol also functions as a substrate for neuronal, and possibly glial, GABA uptake systems.^{7,11} Following ingestion of *A. muscaria*, the acidic gastric microenvironment causes ibotenic acid to be continually decarboxylated, which quantitatively yields muscimol. Thus, the clinical signs seen in the subacute

phase of pantherina–muscara toxicosis are predominantly reflective of the depressant actions of muscimol, and primarily consist of sedation and somnolence, which may be associated with hallucinations and vivid dreams in humans.

Previous veterinary reports of mushroom poisoning are likely rare because of the difficulty associated with establishing a definitive diagnosis.⁹ An empirical diagnosis is usually made based on historical evidence of mushroom exposure and ingestion in combination with concurrent clinical signs. Visualization of ingested mushrooms obtained from vomitus or gastric lavage fluid further supports the diagnosis, but definitive taxonomic identification of mushroom samples obtained in this manner can be very difficult, and may require mycological expertise that is not readily available. Preservation of mushrooms retrieved from ingesta for future identification is best achieved by wrapping samples in paper towels and storing them in a paper bag.⁹

As demonstrated in this study and previous human reports of *Amanita* species toxicoses, metabolites of the incriminated mushroom species can be identified in a variety of body tissues and fluids using HPLC, which allows for the definitive diagnosis of mushroom poisoning.¹² Ion-interaction HPLC techniques, similar to the one described here, have several advantages over other methods, such as paper chromatography (PC) and gas chromatography-mass spectrometry (GC-MS), that have been previously employed to detect ibotenic acid and muscimol in suspected cases of pantherina–muscara poisoning. Ion-interaction HPLC techniques, that have been demonstrated to be sensitive for the simultaneous detection of both metabolites, are readily and economically performed with equipment available in most toxicology laboratories and require minimal sample preparation when compared to PC and GC-MS.^{7,10–12} In humans, ibotenic acid and muscimol appear in urine in quantities detectable by ion-interaction HPLC within 1 hour of mushroom ingestion, which makes urine the preferred test substrate for toxic metabolite detection.^{7,12} Unfortunately, commercial veterinary diagnostic laboratories do not routinely offer diagnostic HPLC services for mycologic toxins.

As few antidotes are available, and antidote administration is recommended only when the specific toxic mushroom species has been identified,^{1,9} general treatment for suspected mushroom poisoning includes gastrointestinal decontamination. The principles of gastrointestinal decontamination have been previously reviewed in detail,¹⁴ but induction of emesis in animals with normal mentation and swallowing reflexes, performance of gastric lavage, and administration of activated charcoal to facilitate adsorption of remaining

toxin are advocated. Activated charcoal was not administered to the patients reported here because of the prolonged interval between toxin exposure and presentation, and the perceived risk of aspiration associated with the CNS signs displayed by both dogs.

Additional symptomatic therapies have been prescribed in humans with pantherina-muscaria poisoning including administration of a variety of anxiolytics, sedatives, and anti-convulsants in cases where seizures are observed. Diazepam, clonazepam, and phenobarbital have been used successfully to treat seizures associated with *A. muscaria* toxicosis in adults and children, as well as in dogs and cats with *A. pantherina* poisoning.⁹ Additional precautionary measures should be employed when benzodiazepines or barbiturates are administered, as both of these classes of drugs are GABA agonists and may act synergistically with muscimol to potentiate CNS depression.^{7,11} Patients receiving benzodiazepines or barbiturates should be monitored closely for respiratory depression which may necessitate mechanical ventilation in some cases. Administration of parenteral diazepam and phenobarbital did not result in any respiratory depression in the 2 dogs reported here, as determined by serial clinical observation and continual pulse oximetry (Dog 1). Administration of methocarbamol largely abated the muscle tremors seen in Dog 1, and was not associated with any significant adverse effects. However, it is possible that the CNS depressant effects of this drug may have acted synergistically with the administered anti-convulsant and delayed the patient's recovery.

The results of this report suggest that the natural history of canine poisoning resulting from ingestion of *A. muscaria* is chronologically and clinically similar to those caused by *A. pantherina* with the notable exception of the acute gastrointestinal distress that preceded the signs of CNS dysfunction in dogs ingesting *A. muscaria*.⁹ Variably severe signs of CNS excitation, caused by the agonistic effects of ibotenic acid on Glu receptors, including stereotypical behaviors, seizures, and muscle tremors, typically develop within 4–12 hours of apparent ingestion of fly agaric and last for 12–24 hours. The final phase of intoxication consists of CNS depression and somnolence, mediated by the GABA-ergic effects of muscimol. Full recoveries typically occur within 24–48 hours when treatments are administered. Based on the outcomes of the dogs reported here, and from previous publications appearing in both the human and veterinary literature, the prognosis for recovery from pantherina-muscaria syndrome should be considered excellent with prompt recognition and appropriate supportive therapy, even in cases with severe clinical signs resulting from ingestion of large numbers of mushrooms.^{3,5,7,9} The diagnosis of panther-

ina-muscaria syndrome can be confirmed by the demonstration of ibotenic acid and muscimol in the urine or serum using HPLC in animals with concurrent clinical signs, in which historical or physical identification of *A. muscaria* or *A. pantherina* species mushrooms is problematic or impossible.

Acknowledgement

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Footnotes

- ^a Diazepam, Abbott Laboratories, North Chicago, IL.
- ^b Atropine, Abbott Laboratories.
- ^c Lactated Ringer's solution, Baxter Animal Healthcare Co., Deerfield, IL.
- ^d Phenobarbital, Elkins-Sinn Inc., Cherry Hill, NJ.
- ^e Propofol, Abbott Laboratories.
- ^f Neurofax EEG 1100, Nihon-Kohden America Inc., Irvine, CA.
- ^g Robaxin, Fort Dodge Animal Health, Overland Park, KS.
- ^h Phenobarbital, Qualitest Pharmaceuticals Inc., Huntsville, AL.
- ⁱ Ibotenic acid, Sigma-Aldrich Co., St Louis, MO.
- ^j Muscimol, Sigma-Aldrich Co.
- ^k Octylamine, ACROS Organics, Morris Plains, NJ.
- ^l Phosphoric acid, J.T. Baker Division of Mallinckrodt Baker Inc., Phillipsburg, NJ.
- ^m Methylene chloride, J.T. Baker Division of Mallinckrodt Baker Inc.
- ⁿ Methanol, J.T. Baker Division of Mallinckrodt Baker Inc.
- ^o Millipore, MilliQ, Milford, MA.
- ^p Speed Vac Model SVC-100H Concentrator, SAVANT Instruments Inc., Farmingdale, NY.
- ^q Beckman Coulter System Gold HPLC, solvent module 126, Model 166 UV-VIS detector, Beckman Coulter Inc., Fullerton, CA.
- ^r 32 Karat, version 5.0, Beckman Coulter Inc.
- ^s Absorbosphere, Alltech, Nicholasville, KY.
- ^t RP-18, Phenomenex, Macclesfield, UK.

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Taking advantage of the experience in ethnomedicinal use of mushrooms: Anti-inflammatory and related pharmacological activities of fly agaric (*Amanita muscaria*) ethanolic extract deserve a modern evaluation

Health benefits of the medicinal use of mushrooms (edible as well as non-edible) have been long recognized by many cultures worldwide [1]. The pharmacological potential of mushrooms, covering antimicrobial, antitumor, immunostimulatory, anti-inflammatory, etc. activities, is today well documented (see [2] for review). This potential has been successfully realized in the development of modern medicinal mushroom preparations, the current sales of which are estimated to be US \$ several billion annually [2].

Amanita muscaria or fly agaric, the strikingly beautiful mushroom, red in color and speckled with white, has been used for several purposes, mostly as a psychostimulant, by different ethnic groups from Mexico to Siberia to Eastern Asia. Whole populations have enjoyed the capacity of fly agaric to kill flies (that is where its common name is from), Mexican and Siberian shamans have used it to mobilize their powers of healing, while Viking warriors have applied it for self-encouragement before the battle [3]. *Amanita muscaria* has also been identified by Wasson [3] as the ancient 'Soma' drink, referred to in ancient Hindu scriptures, the Vedas, which are probably the world's oldest religious text that is still in use. Other stories about magic powers of *Amanita muscaria* also do not lose their popularity, 'Alice in wonderland' being an example.

Slavic nations have their own traditions of *Amanita muscaria* use [4]. Ethnic people are especially fond of the beneficial effects they achieve from the topical application of fly agaric ethanolic (or strong vodka) extract; the home-made extracts have a long history of their use for reduction of the consequences of inflammatory processes in

cases of rheumatic diseases, body injuries, insect bites, etc. It may be of interest to add that, when attending numerous scientific meetings on biomedical research in Russia, Byelarus, and Ukraine, we have often used to ask the outstanding scientists for their opinion of the fly agaric ethanolic extract and the answer was usually: 'it works perfectly'.

Ethanolic/methanolic extracts of several mushrooms have recently been examined for their anti-inflammatory effects by using up-to-date techniques and the results are rather promising (see [2] for review). Bearing the above said (as well as the advances in chemistry of the genus *Amanita* [5]) in mind, we would like to propose *Amanita muscaria* ethanolic extract to be the next one re-examined. We also see prospects for a more complex programme targeted at a modern evaluation of beneficial effects of mushroom extracts, hot water extracts [6] including.

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Peroxisome proliferator-activated receptor gamma: A common link for non-alcoholic fatty liver disease and colorectal cancer?

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a member of the nuclear receptor super-family of ligand-dependent transcriptional factors [1]. PPAR γ plays a role both in colon non-alcoholic fatty liver disease and cancer [2]. It has been shown that PPAR γ plays an important role in the differentiation of adipocytes and monocytes/macrophages, as well as in cell proliferation, apoptosis and carcinoma cell arrest [1]. PPAR γ was cloned as a transcription factor involved in fat cell differentiation and is required for the induction of adipocyte differentiation. PPAR γ induces hepatic steatosis, and liganded PPAR γ promotes adipocyte differentiation [3]. PPAR γ is highly expressed in human colon and colorectal cancer. Structurally, diverse PPAR γ agonists inhibit the growth of multiple cancer cell lines, including colon cancer cells [1].

By the light of these findings, we hypothesize that both non-alcoholic fatty liver disease and colon cancer may result from distorted mechanisms of PPAR γ regulation. PPAR γ ligand effects may be governed by specific molecular genetic determinants and identifying these genetic determinants is therefore of considerable importance [3] to understand the pathogenesis of both non-alcoholic fatty liver disease and colon cancer. Hence, the identification of genetic variants of PPAR γ regula-

tion may not only disclose mechanisms underlying non-alcoholic fatty liver disease development, but may also lead to the discovery of genes that predispose to colorectal malignancy.

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Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*

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- The interaction between the mycorrhiza helper bacteria *Streptomyces* nov. sp. 505 (AcH 505) and *Streptomyces annulatus* 1003 (AcH 1003) with fly agaric (*Amanita muscaria*) and spruce (*Picea abies*) was investigated.
- The effects of both bacteria on the mycelial growth of different ectomycorrhizal fungi, on ectomycorrhiza formation, and on fungal gene expression in dual culture with AcH 505 were determined.
- The fungus specificities of the streptomycetes were similar. Both bacterial species showed the strongest effect on the growth of mycelia at 9 wk of dual culture. The effect of AcH 505 on gene expression of *A. muscaria* was examined using the suppressive subtractive hybridization approach. The responsive fungal genes included those involved in signalling pathways, metabolism, cell structure, and the cell growth response.
- These results suggest that AcH 505 and AcH 1003 enhance mycorrhiza formation mainly as a result of promotion of fungal growth, leading to changes in fungal gene expression. Differential *A. muscaria* transcript accumulation in dual culture may result from a direct response to bacterial substances.

Key words: acetoacyl coenzyme A synthetase, *Amanita muscaria*, cyclophilin, ectomycorrhiza, mycorrhiza helper bacteria, streptomycetes, suppression subtractive hybridization (SSH).

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Introduction

The majority of plants form symbiotic associations, termed mycorrhizas, with filamentous fungi. Mycorrhizal symbiosis improves plant nutrient and water uptake, while the fungal partner gains carbohydrates from its host plant (Smith & Read, 1997). Bacteria have been visualized inside mycorrhizas and as colonies on soil-colonizing fungal hyphae (Foster & Marks, 1966; Nurmiäho-Lassila *et al.*, 1997), and the plant partner selects for bacterial strains beneficial for the symbiosis and for the plant (Frey-Klett *et al.*, 2005). Specifically, the growth of ectomycorrhizal fungi and mycorrhiza formation are promoted by some of the mycorrhizosphere bacteria, termed ‘mycorrhiza helper bacteria’ (MHB; Garbaye, 1994).

MHB have predominantly been reported from fluorescent pseudomonads (Garbaye & Bowen, 1989; Founoune *et al.*, 2002) and from *Burkholderia* and *Rhodococcus* (Poole *et al.*, 2001).

Actinomycetes are known for their capacity to control plant diseases. A number of investigations have reported antagonism of soil actinomycetes to root pathogenic fungi (Rose *et al.*, 1980; Crawford *et al.*, 1993), and that streptomycetes in particular provide a rich source of antifungal compounds (Mousavi & Robson, 2004). When 12 actinomycete isolates were tested for their effects on mycelial growth of ectomycorrhizal fungi (Richter *et al.*, 1989), the bacterial isolates inhibited, promoted or showed no significant effects on hyphal extension in dual culture (DC). Three fungal species

were tested in DC, *Laccaria bicolor*, *Laccaria laccata* and *Thelephora terrestris*, of which the slowest growing fungus, *T. terrestris*, was most sensitive to both growth-promoting and antagonistic actinomycetes (Richter *et al.*, 1989). Some antagonistic actinomycetes are also producers of plant growth-promoting substances, helping plants to withstand adverse conditions and attacks by pathogens (Igarashi *et al.*, 2002). Thus, the opportunity exists to select for actinomycetes stimulatory to certain symbiotic fungi and plant growth but antagonistic to pathogenic organisms.

We have isolated a collection of actinomycetes from the hyphosphere of a spruce (*Picea abies*) stand (Maier, 2003). One of these isolates, *Streptomyces* sp. nov. 505 (AcH 505), significantly increases the mycelial growth and mycorrhiza formation rate of *Amanita muscaria* but suppresses the mycelial extension of the plant pathogens *Armillaria obscura* and *Heterobasidion annosum* (Hampp & Maier, 2004; Maier *et al.*, 2004). In contrast to AcH 505, the second MHB isolated, *Streptomyces annulatus* 1003 (AcH 1003), does not affect the growth of the tested plant pathogenic fungi.

The aim of the experiments presented in this study was to compare the fungus specificity of AcH 505 to that of AcH 1003, and to test the MHB function of these bacteria in a seminatural perlite-moss culture system. Given the present lack of information regarding fungal genes expressed in response to the interaction with MHB, we have used suppression subtractive hybridization (SSH; Diatchenko *et al.*, 1996) to identify genes of *A. muscaria* that are preferentially expressed in DC with AcH 505. For subtraction we used the first 1 cm from the actively growing hyphal front of 9-wk-old DC, as AcH 505 most strongly promotes mycelial growth at this stage. Here we report several *A. muscaria* genes that are differentially transcribed in DC with the bacterium AcH 505.

Materials and Methods

Influence of streptomycetes on fungal growth in axenic conditions

The conditions for bacterial and fungal single and dual cultures, and the screening of bacterial effects on fungal growth, were essentially as described in Maier *et al.* (2004). *Amanita muscaria* growth-promoting *Streptomyces* sp. nov. 505 (AcH 505) and *Streptomyces anulatus* (Beijerinck) Waksman 1003 (AcH 1003), and the control strain *Streptomyces argenteolus* (Tresner *et al.*, 1961) 504 (AcH 504) exerting no effect on hyphal growth in DC (Maier, 2003), isolated from the hyphosphere of *Picea abies* (L.) Karst (Norway spruce) in Haigerloch, Germany (Maier *et al.*, 2004), were cultivated on ISP-2 agar (Shirling & Gottlieb, 1966) in the dark at 20°C.

Amanita muscaria (Fr.) Hooker strains 6 and MEII, isolated from fruiting bodies collected from the Schönbuch forest near Tübingen, Germany, *Amanita muscaria* strain CS83 (Schaeffer, 1995), *Hebeloma cylindrosporum* Romagnesi strain H1-H7

(Debaud & Gay, 1987), *Paxillus involutus* (Batsch: Fr.) Fr. strain ATCC 200175, originally isolated from a fruiting body associated with 15–30-yr-old *Betula pendula* trees growing on coal waste in Midlothian, UK, and *Suillus bovinus* (L.: Fr.) O. Kuntze strain K3 (Sen, 1990) were cultivated in the dark at 20°C on MMN (modified Melin–Norkrans medium) agar (Molina & Palmer, 1982) with 10 g l⁻¹ glucose. Cultures for growth experiments were started with 4-mm-diameter inoculates, obtained from the actively growing rims of single fungal colonies placed in the centre of a sterile cellophane sheet (exclusion limit 10 kDa; Folia, Wendelstein, Germany) on MMN agar (Maier *et al.*, 2004).

The bacterial effect on fungal growth was tested in DC without direct contact between fungus and bacterium on MMN medium [see Maier *et al.* (2004) for details]. A single thin streak (4 cm) of bacteria, obtained from 1-wk-old culture, was applied to the edge of a Petri dish (outside the cellophane sheet) with a sterile inoculation loop. Ten replicates were used. Culture periods were between 6 and 11 wk, depending on the mycelial growth rate of the fungal species in question. At harvest, the first 1 cm of mycelium was rapidly scraped off the cellophane layer, and the material was immediately frozen in liquid nitrogen and stored at –80°C.

Suspension culture experiments

Fungal suspension cultures were started from actively growing hyphae which were finely cut, transferred to MMN medium, homogenized and incubated at 20°C on a rotary shaker at 80 rpm. The MMN medium was exchanged once a week. Carbon-based induction of *A. muscaria* acetoacyl coenzyme A synthetase (*AmAacs*) expression was investigated according to Nehls *et al.* (1999). For *A. muscaria* cyclophilin 40 kDa (*AmCyp40*) induction experiments, suspension cultures of *A. muscaria*, grown for 5 d after MMN medium exchange, were used. Streptomycete suspension cultures were started by transferring an appropriate amount of 1-wk-old bacterial culture to MMN medium and grown for 7 d.

For DC, 10 ml of a filtered (0.22-µm mesh) cell-free 7-d-old streptomycete culture supernatant, taken at an optical density of 600 nm of 0.2, was added to 15 ml of a fungal suspension culture and grown for 3 h. An untreated fungal suspension culture served as a control. All suspension cultures were kept at 20°C on a rotary shaker at 80 rpm. At harvest, suspension cultures were filtered through 100-µm mesh nylon membranes. The material was immediately frozen in liquid nitrogen and stored at –80°C.

Synthesis of ectomycorrhizas and analysis of seedlings

Seeds of Norway spruce (*P. abies*) were obtained from Staatsklänge Nagold (Nagold, Germany) and those of Scots pine (*Pinus sylvestris* L.) from Forelia (Häkkilä, Finland). Seedlings were cultivated until 4 wk old according

to Schaeffer *et al.* (1996). Ectomycorrhizas were synthesized using a Petri dish method modified from Poole *et al.* (2001). Moss peat (Botanical Garden, Tübingen, Germany) and perlite (Knauf Perlite GmbH, Dortmund, Germany) were mixed in a 1 : 1 ratio, crushed, moistened with double-distilled water (10 g water : 15 g mixture) and autoclaved. A slit was cut into the side of the Petri dishes and their cover using a hot scalpel. One part MMN medium (without glucose) was mixed with 4 parts peat–perlite mixture and evenly spread onto the Petri dishes. Plants and fungal inocula (*A. muscaria* 6 or *S. bovinus* K3) were positioned according to Poole *et al.* (2001). Briefly, plants were inserted into the slit, with the root systems placed on the peat–perlite mixture and the shoots exposed to the atmosphere. Three fungal inocula, consisting of 5-mm-diameter discs cut from the growing margin of an MMN agar culture, were placed upside-down along each side of the root at 15-mm intervals, at a distance of 5 mm from the root. For bacterial inoculations, 1-wk-old AcH 505 or AcH 1003 culture in MMN medium was centrifuged (1500 *g* for 15 min) and the pellets were resuspended at OD 0.6 in MMN medium without glucose. Two 500- μ l aliquots of bacteria were pipetted between the roots and the fungal plugs. Four-month-old seedlings were harvested for analysis. For all inoculations, 10 replicates were used. Significance was analysed with Student's *t*-test.

Nucleic acid extractions and construction of a subtracted cDNA library

The first 1 cm of the actively growing rims of 9-wk-old *A. muscaria* 83 mycelia inoculated with AcH 505 was used as starting material for cDNA subtraction. Total RNA from *A. muscaria* was extracted according to Nehls *et al.* (1998). cDNA was synthesized from 0.2 μ g of total RNA (Superscript II; Invitrogen, Groningen, the Netherlands) using a Smart PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA) and then PCR-amplified for 17–18 cycles using conditions recommended by the manufacturer (Clontech). Suppression subtractive hybridization (SSH) was performed between *A. muscaria*–streptomycete 505 DC and *A. muscaria* pure culture using the PCR-Select cDNA Subtraction Kit (Clontech). Subtracted gene fragments were cloned into the TOPO TA cloning vector and transformed into TOPO cells (Invitrogen).

Dot blot analysis of the subtracted library

Samples of bacterial cultures were used for PCR amplification of cDNA inserts from the subtracted library with the primers sense, 5'-TCGAGCGGCCGCCCCGGCAGGT-3' and antisense, 3'-AGCGTGGTCGCGGCCGAGGT-5'. The conditions for the PCR reaction were as follows: 98°C for 3 min, and 30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 2 min. cDNA inserts (200) from randomly selected clones were arrayed on duplicate positively charged nylon membranes (Roche,

Mannheim, Germany). For probe synthesis, digoxigenin-UTP (DIG) labelled total cDNA was amplified from 100 ng total cDNA from *A. muscaria*–AcH 505 DC or *A. muscaria* pure culture using the Smart PCR cDNA synthesis kit (Clontech). The strength of the DIG label in the cDNA populations was equalized by dilution series to allow semiquantitative comparisons of hybridization intensities between filters. The dots were quantified using Quantity One software (Bio-Rad, Munich, Germany). Hybridizations were performed at 68°C for 16 h, and membranes were treated according to the manufacturer's instructions (Roche). Array analyses were replicated two times using first-strand cDNA from two independent cultures. The Thermo Sequence fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Freiburg, Germany) was used to obtain sequence information from differentially hybridized inserts. Software for the sequencing was BASE IMAGIR 4.0 (Li-Cor, MWG-Biotech, Ebersberg, Germany). DNA sequences we compared with already known entries using the BLASTX algorithm (Altschul *et al.*, 1990) of NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>).

Rapid amplification of cDNA ends (RACE) PCR

The SMART RACE cDNA Amplification Kit (Clontech) was used according to the manufacturer's instructions. The *Aacs* cDNA was completed using the gene-specific primer 5'-TGCCGCTGGCCCGATAAGATAGC-3' for 3'-RACE. Primers for *Cyp40* RACE reactions were 5'-CCTCTATGGTATAA-GGGAAG-3' for 3'-RACE and 5'-CCTCGAATGATGAA-CAGGGAAACC-3' for 5'-RACE. All RACE reactions were conducted using the respective primers for 3'- and 5'-RACE provided by the SMART RACE cDNA Amplification Kit and under standard PCR conditions.

Fragments obtained were cloned into pCR2.1 according to instructions given for the TOPO TA Cloning Kit (Invitrogen).

Nucleic acid hybridizations

For northern blot analyses, 2–3 μ g of total RNA was separated on a 1% agarose gel in the presence of formamide and formaldehyde in 20 mM MOPS [3-(4-Morpholino) propane sulphonic acid] buffer, and transferred to a nylon membrane. To confirm dot blot results, membranes were hybridized with DIG-labelled cDNA inserts from the subtracted library. For gene-specific hybridizations, DIG-labelled PCR fragments were obtained from the noncoding 3' regions, using the forward primer 5'-AACTGCCGGAAAA-TTCTC-3' and the reverse primer 5'-TTTCCCATGTCTT-GTTCC-3' for *AmCyp40*, and the forward primer 5'-CATCCTGTTCGTCAAGC-3' and the reverse primer 5'-GCTGGGGCAGCTTAC-3' for *AmAacs*, with the following PCR programme: 94°C for 3 min; 32 cycles of 94°C for 30 s, 46°C for 30 s and 72°C for 2 min; 72°C for 5 min. PCR-based

Table 1 Cyclophilin (Cyp) and acetoacyl coenzyme A synthetase (Aacs) homologues from the genomic DNA sequences of *Phanerochaete chrysosporium* and *Coprinus cinereus*

Designation	Organism	Scaffold	Position (bp)	Theoretical isoelectric point	Theoretical MW (kDa)	% identity to <i>Amanita muscaria</i> protein
AmCyp40	<i>Amanita muscaria</i>			5.77	40 735	
CcCyp40	<i>Coprinus cinereus</i>	11	48300–50100	5.39	40 769	70
PcCyp40	<i>Phanerochaete chrysosporium</i>	102	74800–77000	5.51	40 222	63
CcCyp18	<i>Coprinus cinereus</i>	17	77100–78100	7.97	18 303	82
PcCyp18	<i>Phanerochaete chrysosporium</i>	44	67000–69000	8.96	17 563	64
AmAacs	<i>Amanita muscaria</i>			6.7	77 738	
CcAacs	<i>Coprinus cinereus</i>	10	748200–751100	6.04	77 153	66
PcAacs	<i>Phanerochaete chrysosporium</i>	89	70350–74200	6.01	78 580	66

The properties of the predicted amino acid sequences are shown in comparison to the deduced *Amanita muscaria* proteins. Cyp40 and Cyp18 indicate cyclophilin proteins with the predicted theoretical molecular weights (MWs) of 40 and 18 kDa, respectively.

labelling with DIG and hybridization procedures under high-stringency conditions were performed according to instructions provided in the PCR DIG Probe Synthesis Kit (Roche).

Amino acid sequence analysis

Web-based algorithms provided by ExPASy Molecular Biology Server (PFSCAN; http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) and NCBI (conserved domain search; Marchler-Bauer *et al.*, 2003) were used to analyse the protein sequences. The predicted full-length protein sequences of AmCyp40 and AmAacs were used for phylogenetic analyses. For the alignments, homologous sequences from other fungi detected by BLASTX were selected. In addition, the genomic DNA sequences of *Coprinus cinereus* (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) and *Phanerochaete chrysosporium* (Martinez *et al.*, 2004) were used to search for homologues using the tBLASTN (AmCyp40 protein vs genomic nucleotide sequence) search. The detected amino acid sequences and their properties in comparison with the predicted *A. muscaria* homologues are shown in Table 1.

The peptidyl-prolyl isomerase domains of cyclophilins and full-length Aacs sequences were used for phylogenetic analyses. Sequences were aligned in ClustalX (Jeanmougin *et al.*, 1998). To estimate the phylogenetic relationships, alignments were analysed using a Bayesian approach based on Markov chain Monte Carlo (MCMC; Larget & Simon, 1999) as implemented in the program MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). Four incrementally heated simultaneous Monte Carlo Markov chains were run over 2×10^6 generations using random starting trees and assuming a percentage of invariable alignment sites with gamma-distributed substitution rates of remaining sites. The amino acid substitution model was sampled from the substitution models implemented in MrBayes. Trees were sampled every 100 generations, resulting in an overall sampling of 20 000 trees. The stationarity of the process was controlled using Tracer software, version 1.0 (Rambaut & Drummond, 2003). The first 8000 trees were

discarded ('burn in') and the remaining 12 000 were used to compute a 50% majority rule consensus tree to obtain estimates of posterior probabilities. Branch lengths were averaged over the sampled trees and scaled in terms of expected numbers of amino acid substitutions per site. To test the independence of the results, the Bayesian MCMC phylogenetic analyses were performed twice for each data set. Resulting MCMC trees were rooted with *Streptomyces coelicolor* Aacs as outgroup for the Aacs tree and with *Streptomyces antibioticus* Cyp18 as outgroup for the cyclophilin tree.

GenBank/EMBL/Swissprot accession numbers for the cyclophilin sequences are as follows. PcCyp40: *P. chrysosporium* (not annotated); AmCyp40: *A. muscaria* (AY773481); CcCyp40: *C. cinereus* (not annotated); ScCyp40: *Saccharomyces cerevisiae* (NP_013317); AtCyp40: *Arabidopsis thaliana* (NP_565381); NcCyp40: *Neurospora crassa* (Q9P3 × 9); SpCyp40: *Schizosaccharomyces pombe* (Q11004); MmCyp40: *Mus musculus* (Q9CR16); HsCyp40: *Homo sapiens* (Q08752); HsCyp18: *H. sapiens* (P62937); MmCyp18: *M. musculus* (P17742); DcCyp18: *Digitalis lantana* (T50769); PcCyp18: *P. chrysosporium* (not annotated); CcCyp18: *C. cinereus* (not annotated); ScCyp18: *S. cerevisiae* (NP_010439); PoCyp18: *Pleurotus ostreatus* (CAD10797); PaCyp18: *P. abies* (CAC81066); StrCyp18: *Streptomyces antibioticus* (P83221). Aacs sequences derived from public databases were as follows. AmAacs: *A. muscaria* (DQ089688); CnAacs: *Cryptococcus neoformans* (CNBE4890); UmAacs: *Ustilago maydis* (UM05131.1); RnAacs: *Rattus norvegicus* (AB026291); AnAacs: *Aspergillus nidulans* (CAE47912); StrcAacs: *S. coelicolor* (AL939108.1); CcAacs: *C. cinereus* (not annotated); PcAacs: *P. chrysosporium* (not annotated).

Results

Effects of bacteria on fungal growth and mycorrhiza formation

The two streptomycete isolates, AcH 505 and AcH 1003, showed similar fungus specificities in axenic culture (Table 2).

Table 2 The effect of streptomycete isolates AcH 505 and AcH 1003 on the mycelial growth of a selection of ectomycorrhizal fungi

Fungal isolate	Colony area (cm ²)		
	Bacterial isolate		
	None	AcH 505	AcH 1003
<i>Amanita muscaria</i> 6	24.6	34.7 (+)	37.04 (+)
<i>Amanita muscaria</i> 83	29.8	36.8 (+)	38 (+)
<i>Amanita muscaria</i> MEII	44.5	48.2	50.3
<i>Suillus bovinus</i> K3	16.6	23.5 (+)*	21.01 (+)*
<i>Paxillus involutus</i> 031	30.3	30	33.7
<i>Hebeloma cylindrosporum</i> H1–H7	35.1	24.2 (–)*	21.7 (–)*

Values followed by a sign are significantly higher (+) or lower (–) than the value corresponding to the control treatment in the same line of the table using Student's *t*-test ($P < 0.001$). Dual culture results (AcH 505 vs AcH 1003) with differing ($P < 0.1$) values are marked with asterisks.

The growth of all three tested *A. muscaria* isolates was promoted, but significant changes in mycelial growth rates were only detected for the slowly growing (6 and 83) strains of this fungus. The extent of response to either AcH 505 or AcH 1003 varied significantly in *S. bovinus* and *H. cylindrosporum*. Growth of the *S. bovinus* K strain was promoted, yet both bacteria suppressed *H. cylindrosporum*. The margins of the fungal colonies remained isodiametric in all culture experiments. A mycorrhiza formation test in a peat–perlite culture system was conducted to determine whether AcH 505 and AcH 1003 increase the mycorrhiza formation rate (Table 3). Consistent with the results on mycelial growth promotion in DC, AcH 1003 promoted mycorrhiza formation more strongly than AcH 505. The number of mycorrhizal second-order lateral roots in Norway spruce was increased with AcH 505 (20%) and with AcH 1003 (39%) in comparison with the control treatment. In pine seedlings the number of mycorrhizal first-order lateral roots was increased by 27% with AcH 505 and by 39% with AcH 1003, and the number of second-order lateral roots was increased by 50% with AcH 505 and by 72% with AcH 1003. Furthermore, AcH 505 induced a significant increase in secondary lateral root formation in pine, and a slight increase in formation of these roots in spruce seedlings. No other significant effects on seedling growth of either plant were recorded.

The use of the cDNA subtraction technique to select preferentially expressed genes

As hyphal growth promotion was found to be an important factor for the MHB effect, we performed an SSH analysis with the aim of isolating fungal genes preferentially expressed in DC, i.e. the genes that are related to the induction of

Table 3 Effects of AcH 505 and AcH 1003 on lateral root and mycorrhiza formation in individual seedlings of Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*)

Bacterial isolate	No. of first-order uninfected lateral roots	No. of second-order uninfected lateral roots	No. of first-order mycorrhizal lateral roots	No. of second-order mycorrhizal lateral roots	Total no. of roots	First-order root mycorrhizal rate (%)	Second-order root mycorrhizal rate (%)	Total Mycorrhizal rate (%)
<i>Picea abies</i> – <i>Amanita muscaria</i> 6								
None	28 (8,23)	31.3 (9,37)	ND	14.3 (4,69)	59.3 (16,79)	ND	45.2 (3,5)	24.3 (2)
AcH 505	28.6 (8,83)	38.5 (19,3)	ND	21 (10,8)	67.1 (24,7)	ND	54.6 (+) (3,34)	29.3 (5,83)
AcH 1003	25.4 (10,9)	40 (20,9)	ND	25.1 (12,5)	65.4 (25,4)	ND	63.5 (+) (5,64)	35.7 (+) (11,2)
<i>Pinus sylvestris</i> – <i>Suillus bovinus</i> K3								
None	15.2 (6,12)	42.9 (16,2)	4.5 (2,12)	4.3 (1,16)	58.1 (19,2)	32.3 (14,3)	10.6 (3,13)	15.8 (3,05)
AcH 505	18.4 (4,88)	95 (52,5)	8.1 (+) (3,25)	14.8 (+) (7,41)	113.4 (+) (55,4)	41.1 (9,06)	15.9 (+) (2,77)	21 (+) (3,8)
AcH 1003	16.4 (3,63)	52.2 (15,82)	7.5 (+) (1,96)	9.3 (+) (1,77)	67.5 (16,6)	45.9 (8,52)	18.3 (+) (2,67)	26.1 (+) (3,14)

Picea abies was grown with *Amanita muscaria* for 12 wk and *Pinus sylvestris* with *Suillus bovinus* K3 for 16 wk. Values significantly higher than the control treatment ($P < 0.01$) are indicated with (+). Mean values of 10 replicates are given, with standard deviations in parentheses underneath.

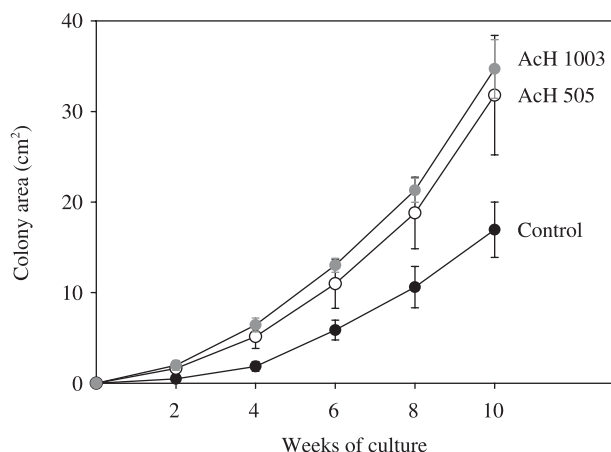


Fig. 1 Time course of *Amanita muscaria* 83 growth induction in dual culture. The colony area of control mycelium (black circles) is compared with that of mycelium in dual culture with AcH 505 (open circles) or with AcH 1003 (grey circles). The interaction with bacteria leads to significantly promoted growth at and after 2 wk of culture, according to Student's *t*-test ($P < 0.01$).

growth of *A. muscaria* hyphae through AcH 505. First, the time course of *A. muscaria* growth promotion was analysed to determine the appropriate stage for mRNA isolations (Fig. 1). The strongest induction with AcH 505 and AcH 1003 was detected between 8 and 10 wk of growth. Nine-wk-old AcH 505-induced *A. muscaria* 83 mycelia were used for gene expression analysis. Differentially expressed sequences were cloned to obtain a subtracted *Am-505* cDNA library. The average size of DNA inserts in the subtracted library was 450 bp.

Altered fungal gene expression in DC

Two hundred randomly picked clones from the subtracted library were analysed by dot blot analysis (data not shown). Fifty-eight clones (28%) showed at least 2-fold increase or decrease in hybridization intensities, 45 of them being up-regulated and 13 down-regulated. Twenty-one of these differentially regulated genes showed statistically significant matches against database sequences (expected value $< e^{-10}$) (Table 4). Differential expression of nine genes was validated by northern analysis. These genes were related to important aspects of the fungus–bacterium interaction, or were unknown genes with significantly altered hybridization intensities. The observed changes in expression rates were comparable to those detected in dot blot analysis (data not shown). For genes of known function, the highest differential expression (6-fold up-regulated) was observed for cyclophilin 40, and the lowest (3-fold down-regulated) for the gamma-aminobutyric acid/polyamine permease. Among the genes of unknown function, clones with strongly increased (6-fold up-regulated; CV632830) and decreased (5-fold down-regulated; DN779411) levels of transcription were found (Fig. 2).

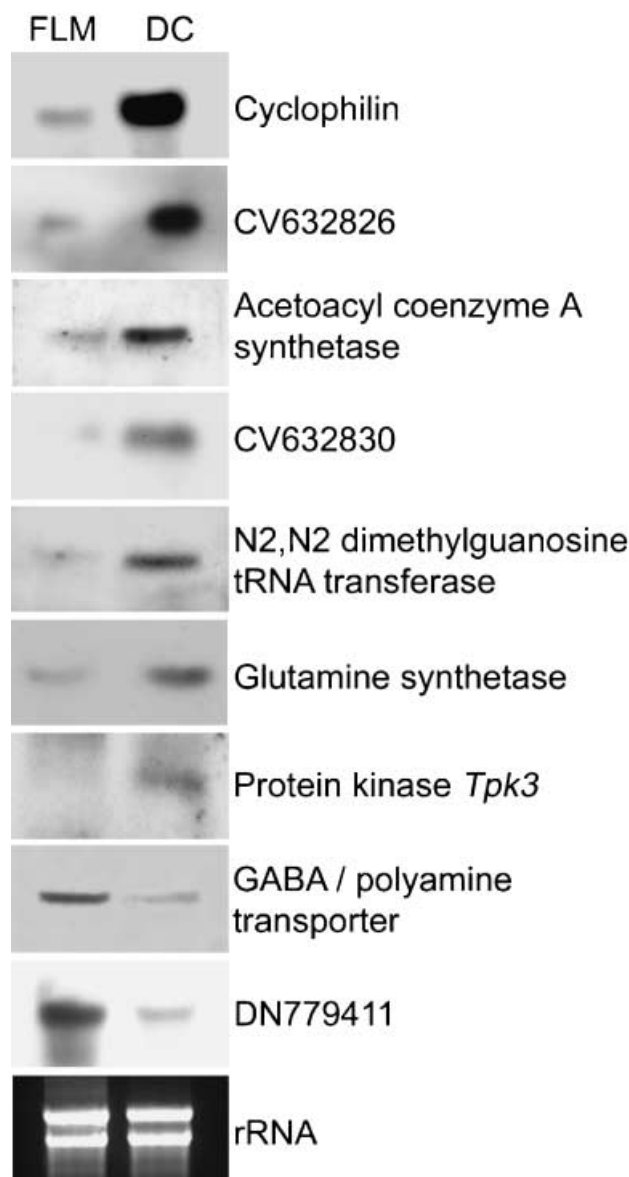


Fig. 2 Transcript levels of subtracted genes in free-living *Amanita muscaria* 83 mycelium (FLM) and in dual culture (DC) with AcH 505 according to northern analysis. Cultures were harvested after 9 wk of growth on MMN medium.

The majority of the genes with known function were related to signal transduction pathways (seven genes; protein kinases and transcription factors) and metabolism (five genes; carbon-metabolism: acetoacetyl coenzyme A synthetase; sulphur-metabolism: adenylyl sulphate kinase and sulphite reductase; nitrogen metabolism: glutamine synthetase and protease). In addition, genes coding for proteins involved in cell growth or cell stress (five genes; cyclophilin 40, gamma-aminobutyric acid/polyamine transporter, ribosomal protein genes and histone H4) and cell structure [two genes; beta-glucosidase and p21-activated kinase (PAK)] were identified.

Table 4 *Amanita muscaria*–AcH 505 interaction-specific library clones

Accession number	cDNA length (bp)	Best database match determined by BLASTX	Species	Expected value	Relative transcript ratio Am–AcH 505/Am
CV632831	537	Hypothetical protein	<i>Coprinus cinereus</i>	8e-18	6
CV632829	564	Peptidyl-prolyl <i>cis-trans</i> isomerase	<i>Neurospora crassa</i>	1e-48	6
CV632830	399	Histone H4	<i>Phanerochaete chrysosporium</i>	9e-37	4
CV632827	697	ND	ND	ND	4
CV632828	490	N2,N2-dimethylguanosine tRNA methyltransferase	<i>Saccharomyces cerevisiae</i>	7e-11	3
CV632826	343	ND	ND	ND	3
CV632832	401	ND	ND	ND	3
CV632847	470	Ribosomal L27e protein	<i>Cryptococcus neoformans</i>	2e-28	2
CV632846	391	Acetoacyl coenzyme A synthetase	<i>Danio rerio</i>	5e-15	2
CV632845	512	Hypothetical protein	<i>Coprinus cinereus</i>	7e-20	2
CV632844	382	Cellulose-binding beta-glucosidase	<i>Phanerochaete chrysosporium</i>	7e-25	2
CV632842	587	Flavin-binding monooxygenase-like	<i>Aspergillus nidulans</i>	7e-21	2
AJ428992	286	Glutamine synthetase	<i>Amanita muscaria</i>	3e-49	2
CV632834	653	Adenylylsulphate kinase	<i>Saccharomyces cerevisiae</i>	1e-49	2
CV632835	471	Ribosomal protein gene	<i>Gibberella zeae</i>	2e-58	2
CV632839	494	Sulphite reductase	<i>Cryptococcus neoformans</i>	1e-58	2
DN779412	645	PAK kinase (Don3)	<i>Ustilago maydis</i>	9e-65	2
CV632841	693	Protein kinase Tpk3	<i>Saccharomyces cerevisiae</i>	1e-18	2
CV632825*	373	Glutamic protease eqolisin; carboxyl peptidase A	<i>Talaromyces emersonii</i>	1e-20	0.5
CV632837*	500	Transcription initiationfactor	<i>Ustilago maydis</i>	3e-16	0.5
CV632840*	266	Reverse transcriptase	<i>Tricholoma matsutake</i>	3e-23	0.5
CV632824*	481	DEAD box RNA helicase	<i>Ustilago maydis</i>	9e-73	0.5
CV632833*	520	Transcription factor	<i>Cryptococcus neoformans</i>	4e-12	0.5
CV632836*	300	Protein kinase	<i>Cryptococcus neoformans</i>	4e-12	0.5
CV632843*	470	γ -aminobutyric acid/polyamine permease	<i>Saccharomyces cerevisiae</i>	7e-28	0.3
DN779411*	211	ND	ND	ND	0.2

The GenBank accession numbers of the nucleotide sequences are given. BLASTX searches gave differing degrees of identity to genes in other organisms (expected values). The first clone has the highest and the last clone the lowest apparent Am–AcH 505/Am transcript ratio, detected by dot blot analysis. Clones marked with an asterisk are less strongly expressed in the interacting fungal hyphae. Am, *Amanita muscaria*; PAK, p21-activated kinase; Tpk3, Protein kinase A catalytic subunit 3.

Two up-regulated genes were selected for further analysis: *Aacs*, to analyse whether the increased *Aacs* expression was related to growth activation of *A. muscaria* mycelia, and *Cyp40*, to investigate whether *Cyp40* expression increased as a result of the addition of bacterial culture supernatants.

Isolation and expression analysis of *Aacs*

Aacs catalyses the activation of acetoacetate, which is needed for efficient sterol biosynthesis (Bergstrom *et al.*, 1984). Increased expression of a functionally related gene, acetyl coA acetyl transferase (*Acat*), was observed in *L. bicolor* hyphae grown in contact with pine root exudates (Podila *et al.*, 2002). We investigated whether the *AmAacs* transcription level was increased as a result of activation of fungal mycelium, as was suggested for *L. bicolor Acat* by Podila *et al.* (2002).

As no previous information about fungal *Aacs* proteins existed, we isolated the full-length *AmAacs* cDNA by RACE. The predicted 702 amino acid *AmAacs* protein sequence

includes two conserved and overlapping regions according to conserved domain search (CD-Search; Marchler-Bauer *et al.*, 2003), namely the AMP-binding enzyme region (amino acids 124–577) and the acyl coenzyme A synthetase/AMP (fatty) acid ligase region (amino acids 81–667). Using the predicted *AmAacs* amino acid sequence, we detected homologue sequences in the published genomes of the homobasidiomycetes *C. cinereus* and *P. chrysosporium* (66% and 66% identity at the amino acid level, respectively), which are both close relatives of *A. muscaria*. Several *AmAacs* homologues were found by BLASTX, such as the hypothetical protein CNBE4890 from the human opportunistic pathogen *C. neoformans* (48% identity), the hypothetical protein UM05131.1 from the maize pathogen *Ustilago maydis* (45%), and the rat *Aacs* (41%; Iwahori *et al.*, 2000). By contrast, no *AmAacs* homologue was found in the budding yeast genome database. The predicted amino acid sequences of the *AmAacs* homologues had comparable molecular weight, pI, AMP-binding and acyl coenzyme A synthetase domain sequences (data not shown). As expected, the homobasidiomycete

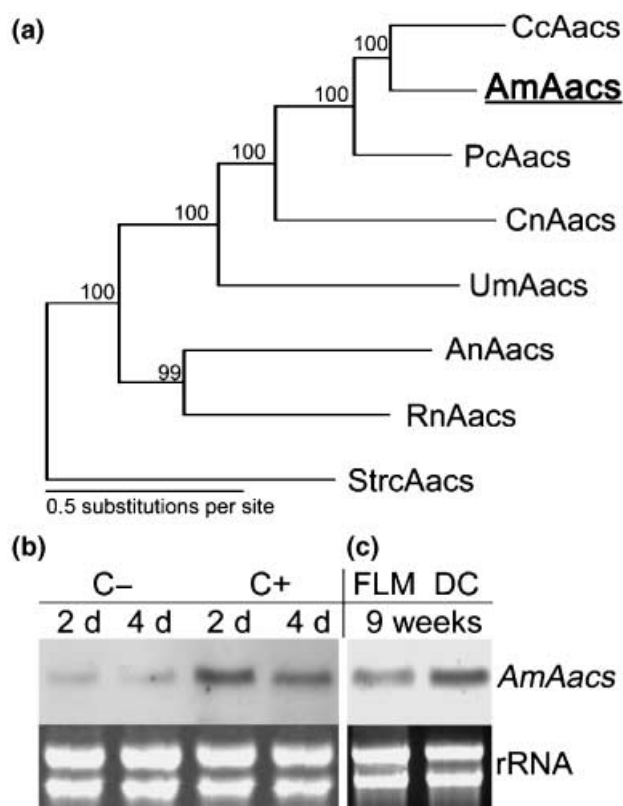


Fig. 3 Phylogenetic and gene expression analysis of *Amanita muscaria* acetoacetyl coenzyme A synthetase (AmAacs). (a) Rooted tree illustrating the phylogenetic relationship between AmAacs and other acetoacetyl coenzyme A synthetases. In the genomes of homobasidiomycetes *Coprinus cinereus* (CcAacs) and *Phanerochaete chrysosporium* (PcAacs), one Aacs homologue was detected according to sequence homology to AmAacs. *Streptomyces coelicolor* Acs (StrcAacs) was included as an outgroup. Other protein sequences used for the analysis were: CnAacs, *Cryptococcus neoformans* Aacs; UmAacs, *Ustilago maydis* Aacs; AnAacs, *Aspergillus nidulans* Aacs; RnAacs, *Rattus norvegicus* Aacs. Bayesian Markov chain Monte Carlo analysis was performed using full-length Aacs sequences. Numbers at branches are *a posteriori* probabilities regarding the monophyly of the respective protein. Branch lengths are proportional to evolutionary distances. The scale bar indicates an evolutionary distance of 0.5 amino acid substitutions per position in the sequence. (b) Carbon-based induction of AmAacs expression. Changes in the transcript level of AmAacs during fungal growth in submerged culture were investigated. No (C-) or 100 mM (C+) glucose was added to mycelia depleted of glucose. Levels of AmAacs gene expression were analysed by northern analysis after 2 or 4 d of culture. (c) For a comparison, the Aacs expression rate at 9 wk of Petri dish culture in free-living mycelium (FLM) and dual culture (DC) is shown.

(*A. muscaria*, *C. cinereus* and *P. chrysosporium*) Aacs proteins grouped together in phylogenetic analysis (Fig. 3a).

We used submerged *A. muscaria* culture to analyse whether the changed AmAacs expression in DC is a result of the activation of dormant hyphae (Podila *et al.*, 2002). The effect of addition of glucose on Aacs expression was determined in *A. muscaria* suspension culture. The glucose-supported (100 mM)

fungal culture showed a 3-fold increase in Aacs expression in comparison with glucose-starved hyphae (Fig. 3b). A 2-fold up-regulation of Aacs expression was observed in DC at 9 wk of culture (Fig. 3c).

Isolation and characterization of Cyp40 cDNA

The Cyp40 proteins are prolyl isomerases, which are necessary for efficient cell growth and endurance of cell stress in *S. cerevisiae* (Duina *et al.*, 1996). Of all subtracted genes of known function, the level of *A. muscaria* Cyp40 transcription is most significantly increased in DC hyphae, and we decided to investigate whether comparable differential expression occurs already in a short-term culture.

Using RACE PCR, the complete cDNA sequence of the first cyclophilin of *A. muscaria* was determined and denoted AmCyp40 for the *A. muscaria* cyclophilin 40-kDa protein. Homology searches using genome sequences or the BLASTX algorithm revealed related proteins belonging to the cyclophilin family, most notably from *C. cinereus* and *P. chrysosporium* genomes (70% and 63% identity at the amino acid level, respectively).

A motif scan of the predicted AmCyp40 protein sequence by PFSCAN detected a cyclophilin-type peptidyl-prolyl *cis-trans* isomerase domain of 166 amino acids. In addition, the profile scan also detected the presence of two putative tetratricopeptide repeat (TPR) motifs for protein-protein interactions and a putative calmodulin-binding site. An analysis using the 'PESTfind' algorithm revealed the presence of two putative PEST sequences. A study of 12 other fungal and mammalian 40-kDa cyclophilins obtained from the GenBank/EMBL database showed that only the fungal cyclophilins contain PEST sequences. TPR motifs are only absent from the 40-kDa cyclophilin wis2 of *S. pombe*, and all investigated proteins contain the putative calmodulin-binding site. A phylogenetic analysis of AmCyp40 based on the conserved peptidyl-prolyl *cis-trans* isomerase region (166 amino acids) of the deduced protein sequence was used for the determination of the phylogenetic position of AmCyp40 among the eukaryotic cyclophilins. Cyclophilins clustered (i) according to the size of the entire protein and (ii) according to the relatedness of organisms. AmCyp40 grouped with the other homobasidiomycete 40-kDa cyclophilins (Fig. 4a).

AmCyp40 is highly expressed in the DC hyphae with both AcH 505 and AcH 1003 (data not shown). In order to determine whether the increase in AmCyp40 transcript is a growth effect, the expression was analysed in short-term experiments in suspension culture. *A. muscaria* suspension cultures were incubated with a cell-free supernatant of the streptomycete strains AcH 505 and AcH 1003 and cultivated for 6 h. Incubation with supernatant of the strain AcH 504, which exerts no effects on *A. muscaria* growth (Maier *et al.*, 2004), served as a control. Levels of AmCyp40 transcription were strongly increased in the presence of AcH 505 and AcH 1003 supernatants after 6 h, while there was no effect with control strain AcH 504 (Fig. 4b).

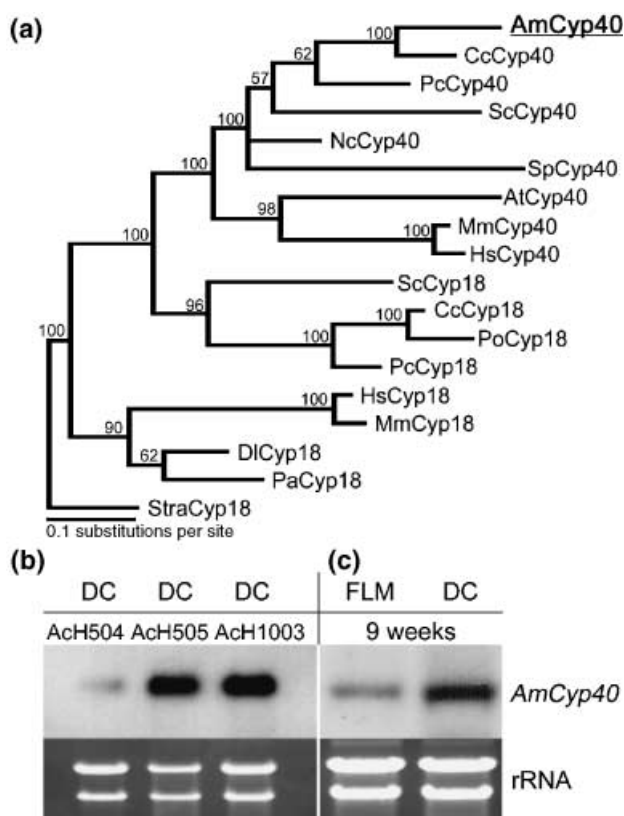


Fig. 4 Phylogenetic analysis and levels of *Amanita muscaria* cyclophilin 40-kDa (*AmCyp40*) gene expression. (a) Phylogenetic analysis of *AmCyp40*. The conserved region of the peptidyl-prolyl *cis-trans* isomerase (PPIase) domain of a collection of 40-kDa and 18-kDa cyclophilins was used to perform Bayesian Markov chain Monte Carlo analysis. Numbers at branches are *a posteriori* probabilities regarding the monophyly of the respective groups of proteins. *Streptomyces antibioticus* cyclophilin is shown as an outgroup (StraCyp18). The cyclophilin homologues from the homobasidiomycetes *Coprinus cinereus* (CcCyp40 and CcCyp18) and *Phanerochaete chrysosporium* (PcCyp40 and PcCyp18) were identified according to sequence homology to *AmCyp40*. The other 40- and 18-kDa cyclophilins used in the analysis were from the following organisms: ScCyp40, *Saccharomyces cerevisiae*; AtCyp40, *Arabidopsis thaliana*; NcCyp40, *Neurospora crassa*; SpCyp40, *Schizosaccharomyces pombe*; MmCyp40, *Mus musculus*; HsCyp40, *Homo sapiens*; HsCyp18, *Homo sapiens*; MmCyp18, *Mus musculus*; DIcyp18, *Digitalis lantana*; ScCyp18, *Saccharomyces cerevisiae*; PoCyp18, *Pleurotus ostreatus*; PaCyp18, *Picea abies*. (b) Short-term induction of *AmCyp40* transcription by AcH 505 and AcH 1003 culture supernatants. *A. muscaria* suspension cultures were incubated for 6 h with culture supernatants of streptomycete strains AcH 504 (supernatant control), AcH 505 and AcH 1003. (c) For a comparison, the level of *AmCyp40* expression at 9 wk of Petri dish culture is represented.

Discussion

The two streptomycete strains investigated in this contribution have similar specificities for ectomycorrhizal fungi. This is surprising, as Maier *et al.* (2004) showed previously that AcH

505 suppresses the growth of *H. annosum* and *Armillaria abietina*, whereas AcH 1003 has no effect on these plant pathogens. This indicates an important application for ectomycorrhiza helper bacteria such as AcH 505: the simultaneous growth promotion of mycorrhizal and growth suppression of pathogenic fungi.

Resistance to antifungal substances can be an important characteristic for fungal species colonizing the rhizosphere (Whipps, 2001). *H. cylindrosporum* is inhibited in its growth and mycorrhiza formation by MHB (*Bacillus*, *Pseudomonas*) associated with *L. laccata* (Garbaye & Duponnois, 1992). Accordingly, the streptomycetes of this study suppressed *H. cylindrosporum* growth in DC experiments, indicating that *H. cylindrosporum* is sensitive to antifungal substances produced by very different bacterial species.

Fungi and bacteria were spatially separated in DC, although a continuum of nutrient solution existed in the Petri dish cultures. The shape of fungal colonies remained isodiametric in all cultures, indicating that the substances that promote (*A. muscaria* and *S. bovinus*) mycelial growth must readily diffuse in the culture medium, more rapidly than the mycelial growth rate of responsive fungi. Such an effect has been previously shown for the ECM fungus *Pisolithus tinctorius*, as even a local application of the small fungal growth-promoting plant secondary substance rutin did not result in asymmetric colony extension of this fungus (Lagrange *et al.*, 2001). Although streptomycetes are known to produce a variety of volatile compounds (Schöller *et al.*, 2002), volatiles from AcH 505 and AcH 1003 did not show any effects on the mycelial extension rates of the six tested fungal isolates. To investigate the differential responses of the fungal species in detail, we are currently analysing mycelial growth-stimulating and -suppressing substances from the AcH 505 culture supernatant.

Differential gene expression in DC

Subtractive hybridization is a well-accepted method to detect differentially regulated genes in ectomycorrhizal fungi. It has been used to select fungal genes expressed preferentially in the presymbiotic phase (Podila *et al.*, 2002), in ectomycorrhizal tissues (Voiblet *et al.*, 2001; Peter *et al.*, 2003) and in extramatrical hyphae (Morel *et al.*, 2005). We present the first detailed report on differential fungal gene expression during DC with mycorrhiza helper bacteria. Becker *et al.* (1999) showed modulation of *in vitro* translation products after inoculation of *L. bicolor* with culture filtrates from a growth-promoting and a growth-suppressing streptomycete strain, but no information concerning the identity of the regulated mRNAs was given in their contribution. The presented data thus identify the first fungal genes with altered expression rates in DC with ectomycorrhiza helper bacteria, as well as providing the first clues to the mechanisms that lead to growth promotion of fungal mycelia. Three aspects of mycorrhiza helper bacteria were chosen for a more detailed analysis:

enhancement of the fungal growth rate, the stress response and signalling between bacteria and fungi.

Accelerated fungal growth and cell stress The presented data show that *A. muscaria* is able to change its gene expression in response to bacterial stimuli. The largest number of induced genes (five) are related to fungal metabolism. These include *AmAacs*, the first acetoacetyl-coA synthetase cDNA isolated from fungi. Aacs activity (Bergstrom *et al.*, 1984) and expression profiles (Ohgami *et al.*, 2003) suggest that this enzyme is involved in cholesterol biosynthesis. We suggest that part of the response of *A. muscaria* to AcH 505 is the induction of increased expression of *AmAacs*, leading to higher production of ergosterol, which is needed for promoted mycelial growth. As carbon, especially glucose, is a key regulator of fungal gene expression in ECM symbiosis (Nehls *et al.*, 1999; Nehls, 2004), the levels of *AmAacs* expression were analysed in carbon-depleted (dormant) and in well-supported (activated) *A. muscaria* suspension cultures. We found an up-regulation of *Aacs* expression in the glucose-supplemented culture, comparable to that seen in DC experiments at 9 wk of growth. After 9 wk of DC, the fungal mycelium nearly covered the Petri dish, and it had probably consumed most of the available glucose. As no agar-degrading activity that might lead to an additional carbon supply has been observed in bacterial cultures, the observation suggests that (i) the fungus is able to change its gene expression in response to bacterial stimuli, and (ii) an effective bacterial compound acting in a similar manner to glucose could be produced by the bacterium.

The transport of polyamines and gamma-aminobutyric acid are mediated by the vacuolar Uga4 permease in budding yeast *S. cerevisiae* (Uemura *et al.*, 2004). The inhibitor of polyamine biosynthesis difluoromethylornithine decreases the mycelial growth rate of *P. involutus* (Zarb & Walters, 1994). The down-regulation of *Uga4* in DC may thus reflect changes in individual polyamine concentrations in the DC hyphae, necessary for the faster growth of fungal mycelia.

Peptidyl-prolyl *cis-trans* isomerases (PPIases) are enzymes that bind to mature proteins to control their activity, subunit assembly and localization (Dolinski & Heitman, 1997). Cyp40 proteins are involved in the regulation of cell proliferation and differentiation and in the control of cell stress. The closest characterized homologues of *AmCyp40* are the yeast Cyp40 family members encoded by *cpr6* and *cpr7* genes. Yeast strains with disrupted *cpr7* show a significant impairment of the rate of cell division, leading to the slow growth phenotype (Duina *et al.*, 1996). The elevation of *AmCyp40* transcription in coculture could thus play a role in the enhanced hyphal growth rate observed in DC. As the streptomycete culture supernatants were added to submerged fungal cultures, an increased level of *AmCyp40* expression was observed with both AcH 505 and AcH 1003 supernatants. By contrast, the use of the culture supernatant of *S. argenteolus* 504, a streptomycete strain exerting no effects on *A. muscaria*

growth, resulted in basal levels of *AmCyp40* expression. This indicates that additional factors, perhaps the presence of stimulatory compounds in the supernatants of AcH 505 and AcH 1003, lead to increased *AmCyp40* expression.

ECM fungi are known to release extracellular enzymes, such as mono-oxidases, capable of oxidizing aromatic (Gramss *et al.*, 1998) and nonaromatic (Takakura & Kuwata, 2003) substances. *A. muscaria* mono-oxidase expression was observed to be increased in DC hyphae, suggesting an activation of specific steps in either secondary metabolism or catabolism in *A. muscaria*.

Signalling between bacteria and fungi Changes in gene expression levels occur in parallel with modulated transcription of signalling-related genes such as PAK and cAMP-dependent protein kinase (PKA). PAK is a central regulator of polarized growth in eukaryotes (Leveleki *et al.*, 2004), and the induced level of PAK expression suggests modulated organization of the actin cytoskeleton in DC hyphae. PKA regulates the cell cycle and metabolism in yeast and mammalian cells. The increased expression level of the gene related to the catalytic subunit of PKA, *Tpk3*, in *A. muscaria* indicates altered growth-related signal transduction in DC.

The differentially expressed genes detected in this *in vitro* approach could serve as a basis for the analysis of *A. muscaria* gene expression in more natural culture systems such as non-sterile microcosms or pot cultures. Analysis of comparable fungal genes in the growth-promoted cultures of *S. bovinus*, and in the growth-inhibited cultures of *H. cylindrosporum* and *H. annosum*, would provide information about the specificity of the fungal response to AcH 505.

Mycorrhiza formation tests

Our findings support the view of Poole *et al.* (2001) that MHB have a ubiquitous distribution, and that the main helper mechanism is fungal growth promotion. This widens the scope of MHB investigations, as previous studies mainly focused on *Bacillus* and *Pseudomonas* species (Garbaye, 1994; Founoune *et al.*, 2002), and resulted in the isolation of MHB from *Burkholderia* and *Rhodococcus* (Poole *et al.*, 2001).

As the ability of the bacteria to stimulate mycelial growth correlated with their effect on mycorrhiza formation, we suggest that the effective mechanism for MHB is predominantly hyphal growth promotion. When *Bacillus* and *Pseudomonas* MHB were investigated, the behaviour of fungal mycelium in DC correlated with mycorrhiza formation tests (Garbaye & Duponnois, 1992; Frey-Klett *et al.*, 1997; Founoune *et al.*, 2002), and Poole *et al.* (2001) suggested that promoted mycelial growth of *Lactarius rufus* is the probable mechanism for the MHB outcome in the Scots pine model system. According to the observations of Richter *et al.* (1989) with *T. terrestris*, the *A. muscaria* strain with the slowest observed growth rate (strain 6) was the most sensitive to the streptomycete isolates

in *in vitro* tests. This suggests that MHB may compensate for slow mycelial growth rates in receptive fungal isolates.

For *A. muscaria*, however, we have observed that the fungal mycelial density decreases in DC with both streptomycete isolates (Maier, 2003; M.T., unpublished data). This suggests either that the observed MHB effect is caused by a faster spreading of fungal mycelia on the peat–perlite culture system (but not by the higher mycelial biomass) or that additional, plant-related factors are involved. As originally suggested by Garbaye (1994), the latter could include increased receptivity of the roots to the fungal hyphae, or improved fungal colonization of the fine roots produced by plant cell wall modifying bacterial substances. According to our results, and a previous study on mycorrhiza formation in Norway spruce on a MMN agar medium conducted by Maier (2003), AcH 505, but not AcH 1003, promotes root branching. Plant growth promoters with auxin-like activity have previously been isolated from *Streptomyces hygroscopicus* (Igarashi *et al.*, 2002).

To conclude, the present data confirm that the streptomycetes AcH 505 and AcH 1003 are MHB, exerting their positive action mainly through the activation of fungal metabolism. As a result of the changed gene expression levels, fly agaric mycelia activated in DC should have a modulated physiology. The question now arises as to which bacteria-generated substances give rise to the improved growth of selected mycorrhizal fungi and inhibit preferentially the growth of phytopathogenic fungi.

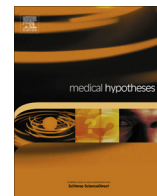
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Mannitol in *Amanita muscaria* – An osmotic blood–brain barrier disruptor enhancing its hallucinogenic action?



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ABSTRACT

Hypothesis have been made that relatively high level of mannitol present in the tissues of fly agaric (*Amanita muscaria*) enables more efficient transportation of these active substances into the brain and thus enhance their total activity. It may have been supported by the fact that hallucinogenic effect after *A. muscaria* consumption is greater than after ingestion of an active substance quantity which the eaten fungi dose contain.

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With its bright red, sometimes dinner plate-sized caps fly agaric (*Amanita muscaria* (L.: Fr.) Lam. s.l.) is one of the most conspicuous member of subgenus *Amanita* and also one of the most striking of all mushrooms [1,2]. This fungus is native to temperate and boreal zones of the Northern Hemisphere; however, it has also been introduced to South Asia, New Zealand, Australia, Tasmania, Hawaii, South America, as well as Central and South Africa [3].

Ancient tribes and civilizations used hallucinogenic fungi to enter the spirit world. The fly agaric may have been the earliest hallucinogenic substance used for religious or shamanic purposes, dating back possibly over 10,000 years [1]. The shamanic preparation and use of the mushroom are meant to induce higher levels of consciousness, vivid visions, spiritual growth, elation and hyperactivity [2]. They also alter the perception of sight, sounds etc. (the senses) and change/enhance the feelings and thoughts of the user [4].

During isolation and separation of *A. muscaria* components we had isolated quite large quantities of mannitol. It was obtained upon fractionation of methanolic extract of dry mushroom by means of flash chromatography as white crystals melting at 166–169 °C (lit. m.p.p. 162–168 °C [5]). The structure mannitol was confirmed by ¹H NMR spectroscopy. This finding is not surprising since mannitol has been already mentioned as prominent component of sugar fraction of fly agaric and other mushrooms [6,7]. Surprising is, however, that this compound accounts of 1% of the dry mass of the mushroom (10–11 mg per 1 g of powdered fly agaric).

The intra-arterial infusion of mannitol has been shown to cause vasodilatation and osmotic shrinkage of endothelial cells and con-

sequently hyperosmotic opening of blood–brain barrier [8–10]. This effect has been even applied to enhance the delivery of drugs into human brains infiltrated by tumors [11–13], management of the cerebral oedema and raised intracranial pressures [11], as well as for curing glaucoma [14]. Mannitol has been also approved as safe nonnutritive sweetener [15].

Mannitol serves as one of the osmoprotectants conferring plant resistance against oxidative stress [16] and salt tolerance and thus its level in plant is strictly dependent on environmental conditions. The level of this polyol in plant tissues varies usually accounts form 0.05–0.5% of dry mass [17–20], with celery producing 1% of this compound upon salt stress [21]. Fungi usually contain higher level of this compound with unbelievable level of 30% of dry mass reported for *Agaricus* sp.[22–24].

In contrast to other *Amanitas*, which produce small diversity of ninhydrin-positive compounds, *A. muscaria* produces around 20–30 of such compounds (amines, amino acids, alkaloids and peptides), a variety of which are suspected to be hallucinogenic. This may explain a unique position of this mushroom in human culture and ethnical medicine [25].

We hypothesize that the high level of mannitol present in the tissues of fly agaric enables more efficient transportation of these active substances into the brain and thus enhance their total activity. This may have found support in that the application of equivalent amounts of ibotenic acid (being the major hallucinogen of *Amanitas*) causes far lower effect than fungi itself [26]. Also the fact that *Amanita pantherina* containing similar amounts of hallucinogens than *A. muscaria* exerts higher hallucinogenic effects [27] might support this assumption, since the level of this polyol in its tissues is four-time higher [6].

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Of course, comparison of effects exerted by *Amanita* mushrooms is only rough since both of them contain cocktails of hallucinogenic substances of different composition. Moreover, the roughness of this comparison is complicated by the fact that psychoactive substances are of variable content depending on the mushroom growth conditions [28].

If so, there is a question if the amount of mannitol in fly agaric tissues is sufficient to exert such an action. This is a difficult to judge since for medication purposes it is used in a form of infusion of high amounts (250–1500 mg) of this compound. Since it is a non-toxic substance its minimal dose required to modify brain-barrier permeability has not been studied. Anyway, in order to reach an active level of hallucinogens the use of 3–6 fly agaric caps is required, which gives 70–200 mg of mannitol, which is quite significant dose.

The action of mannitol is additionally complicated by its diuretic activity, which may result in clearance of the hallucinogens from the body. However, in order to receive such an effect massive (gram) intravenous doses of this compounds are used [29,30], which seems to eliminate the role of diuresis in *Amanita* intoxication.

Conflict of interest statement

None.

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Beringian origins and cryptic speciation events in the fly agaric (*Amanita muscaria*)

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Abstract

Amanita muscaria sensu lato has a wide geographic distribution, occurring in Europe, Asia, Africa, Australia, New Zealand, and North, Central and South America. Previous phylogenetic work by others indicates three geographic clades (i.e. 'Eurasian', 'Eurasian-alpine' and 'North American' groups) within *A. muscaria*. However, the historical dispersal patterns of *A. muscaria* remained unclear. In our project, we collected specimens from arctic, boreal and humid temperate regions in Alaska, and generated DNA sequence data from the protein-coding beta-tubulin gene and the internal transcribed spacer (ITS) and large subunit (LSU) regions of the ribosomal DNA repeat. Homologous sequences from additional *A. muscaria* isolates were downloaded from GenBank. We conducted phylogenetic and nested clade analyses (NCA) to reveal the phylogeographic history of the species complex. Although phylogenetic analyses confirmed the existence of the three above-mentioned clades, representatives of all three groups were found to occur sympatrically in Alaska, suggesting that they represent cryptic phylogenetic species with partially overlapping geographic distributions rather than being allopatric populations. All phylogenetic species share at least two morphological varieties with other species, suggesting ancestral polymorphism in pileus and wart colour pre-dating their speciations. The ancestral population of *A. muscaria* likely evolved in the Siberian–Beringian region and underwent fragmentation as inferred from NCA and the coalescent analyses. The data suggest that these populations later evolved into species, expanded their range in North America and Eurasia. In addition to range expansions, populations of all three species remained in Beringia and adapted to the cooling climate.

Keywords: biogeography, coalescent, fungi, nested clade analysis, phylogeography, supertree

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Introduction

Amanita muscaria (L.: Fr.) Hooker, the 'fly agaric', is probably the most famous and most illustrated fungus and embodies the concept of 'mushroom' in many cultures. Its popularity likely arises from its attractive appearance, wide geographic distribution, and perhaps from its psychoactive properties (Benjamin 1995; Hudler 1998; Michelot & Melendez-Howell 2003). There are several varieties, primarily described to distinguish the different colour forms, such as *A. muscaria* var. *muscaria* (L.: Fr.) Hooker (pileus red, stem and warts white), *A. muscaria* var. *alba* Peck (pileus, warts

and stem white to tan), *A. muscaria* var. *flavivolvata* (Singer) Jenkins (pileus orange to red, warts tannish-yellow, stem white to cream), *A. muscaria* var. *formosa* (Pers.: Fr.) Bertillon in DeChambre (pileus orange to yellow, warts and stem yellowish to tannish), *A. muscaria* var. *persicina* Jenkins (pileus melon, warts tannish to yellowish), and *A. muscaria* var. *regalis* (Fr.) Bertillon in DeChambre (pileus brown, warts tannish to yellowish) (Jenkins 1986). *A. muscaria* is native to temperate or boreal forest regions of the Northern Hemisphere; however, it has been introduced to New Zealand, Australia, South America, and South Africa (Reid 1980; Thiers 1982; Santiago *et al.* 1984; Jenkins 1986; Tan & Wu 1986; Pérez-Silva & Herrera 1991; Reid & Eicker 1991; Ridley 1991; Rimóczi 1994; Tulloss *et al.* 1995; Bhatt *et al.* 2003). It is an ectomycorrhizal

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(ECM) fungus with a wide host range (Trappe 1962). Although it is most commonly associated with various birch (*Betula*), pine (*Pinus*), spruce (*Picea*), fir (*Abies*) and larch (*Larix*) species, it is known to form ECM associations with representatives of other genera, particularly when its primary hosts are rare or nonexistent in a certain area. For example, after being introduced to the Southern Hemisphere by pine seedlings transported from Europe, it has been observed to form ECM symbioses with native trees, such as *Nothofagus*, *Kunzea* and *Leptospermum* species (Bagley & Orlovich 2004). Also, at least one morphological variety, *A. muscaria* var. *regalis*, occurs above altitudinal tree line in interior Alaska, where it has been found associated with *Dryas* and *Salix* species (Miller 1982).

Prior research in the literature suggests that *A. muscaria* exhibits substantial variation in morphology and toxin content (Benedict 1966; Jenkins & Petersen 1976; Jenkins 1986). Despite the broad awareness of the plasticity of *A. muscaria* across different geographic regions, Oda *et al.* (2004) were the first authors to report on the phylogeny and biogeography of the species complex based on DNA sequence data generated from specimens collected in Japan, Nepal, New Zealand, Norway, Poland, the United Kingdom, and various parts of the United States. They found three distinct clades in *A. muscaria* that they considered 'Eurasian', 'Eurasian subalpine' and 'North American' groups, corresponding to geographic differences (i.e. allopatric populations). They hypothesized that the ancestral group of *A. muscaria* existed only in Eurasia and later migrated to North America via land bridges.

Beringia, including Alaska and northeastern Siberia, has long been a focal point for biogeographic research in a wide range of plant and animal taxa. This high level of interest arises for two principal reasons. First, due to its diverse landscape and climate and the fact that much of the region remained ice-free during glacial maxima, Beringia served as a refugium for arctic and sub-arctic flora and fauna. Second, during much of the Tertiary and the Quaternary periods, Beringia was the major land connection between Asia and North America and provided migration routes to a wide variety of organisms (for example, see Adams & Faure 1997; Qian 1999; Elias 2000; Swanson 2003; Kaufman *et al.* 2004). Despite the importance of the unique biogeographic history of Alaska, no specimen of *A. muscaria* has been investigated from this region. Therefore, our goal was to further elucidate the phylogenetic and phylogeographic structure in *A. muscaria* by collecting and analysing specimens from Arctic, boreal and humid temperate regions in Alaska. We generated DNA sequence data from the protein-coding beta-tubulin gene and the internal transcribed spacer (ITS) and large subunit (LSU) regions of the ribosomal DNA repeat, and conducted comprehensive phylogenetic analyses including homologous *A. muscaria* sequences published by Oda *et al.* (2004). We used genea-

logical concordance as outlined by Taylor *et al.* (2000) to determine phylogenetic species boundaries within *A. muscaria*. We conducted phylogenetic analyses based on individual data sets for each locus, on a combined data set of the three loci, and using a 'phylogenetic supertree' approach (Sanderson *et al.* 1998). In addition, we used nested clade analyses (NCA) (Templeton 1998) to reveal the phylogeographic history of the individual phylogenetic species and the species complex as a whole. To be able to better interpret and place in time the results of the phylogeographic analyses, we estimated the ages of the divergence points of the main clades using molecular clock methods. Also, we conducted coalescent-based simulations of genealogical relationships to further enhance the precision of estimates of population and mutation ages, migration, and mutational structures of ancestral populations (Beerli & Felsenstein 1999; Nielsen & Wakeley 2001; Carbone *et al.* 2004).

Materials and methods

Isolates and DNA extraction

Twenty specimens were collected from various geographic regions of Alaska (Table 1). Sporocarps were deposited in the University of Alaska Fairbanks (UAF) Mycological Herbarium. DNA was extracted from small samples of dried specimens using the E-Z 96® Fungal DNA Kit (Omega Bio-tek). ITS and beta-tubulin sequences of additional *Amanita muscaria* isolates were downloaded from GenBank (Table 1). Homologous sequences of *Amanita pantherina* (isolate FB-30958) published by Oda *et al.* (2004) were used to root all trees.

PCR and DNA sequencing

A portion of the beta-tubulin gene was amplified in polymerase chain reaction (PCR) mixtures containing 16.5 µL PCR water, 2.5 µL 10 × PCR buffer (0.5 M KCl, 0.1 M Tris-HCl pH 8.3, 0.025 M MgCl₂), 2.5 µL 10 × dNTPs (2 mM of each dNTP), 0.125 µL *Taq* DNA polymerase (Fisher Scientific), 0.25 µL of 10 µM forward primer and reverse primer, and 1 µL template DNA (original DNA solution extracted). PCR and cycle sequencing reactions were performed in a PTC-220 thermocycler (Programmable Thermal Controller) using primers and settings specified by Oda *et al.* (2004). Amplification products were electrophoresed in a 1.0% agarose gel and stained with ethidium bromide for visualization of the bands. PCR products were purified directly using the QIAquick® PCR Purification Kit (QIAGEN). Purified amplification products were sequenced using the Applied Biosystems (ABI) BigDye® version 3.1 Terminator Kit and an ABI 3100 automated capillary DNA sequencer (PerkinElmer).

Table 1 *Amanita muscaria* isolates included in the multilocus phylogenetic analyses

	Isolate code*	Origin	GenBank Accession no.		
			ITS	beta-tubulin	LSU
<i>A. muscaria</i>	GAL2814	Dalton Highway, mile 122, Alaska, USA	DQ060897	DQ060917	DQ060877
	GAL4302	Juneau, Alaska, USA	DQ060910	DQ060923	DQ060890
	GAL5895	Nome, Alaska, USA	DQ060898	DQ060918	DQ060878
	GAL5900	Nome, Alaska, USA	DQ060902	—	DQ060882
	GAL5946	Nome, Alaska, USA	DQ060903	—	DQ060883
	GAL8950	Denali National Park, Alaska, USA	DQ060901	—	DQ060881
	GAL15776	Bonanza Creek LTER site, Alaska, USA	DQ060893	DQ060913	DQ060873
	30961†	Aomori-shi, Aomori, Japan	AB080980	AB095892	—
	30962†	Kitakoma-gun, Yamanashi, Japan	AB080981	AB095893	—
	30963†	Kitakoma-gun, Yamanashi, Japan	AB080982	AB095894	—
	30976†	Kiso-gun, Nagano, Japan	AB081294	AB095895	—
	30977†	Ohno-gun, Gifu, Japan	AB081295	AB095896	—
	30985†	Ohno-gun, Gifu, Japan	AB096048	AB095897	—
	30978†	Chino-shi, Nagano, Japan	AB081296	AB095858	—
	30981†	Chino-shi, Nagano, Japan	AB096049	AB095859	—
	30982†	Chino-shi, Nagano, Japan	AB096050	AB095860	—
	30964†	Gdynia, Poland	AB080983	AB095899	—
	30965†	Gdansk, Poland	AB080984	AB095900	—
	31452†	Hampshire, England, UK	AB080777	AB095901	—
	31445†	Surrey, England, UK	AB080778	AB095902	—
	80048†	Surrey, England, UK	AB080779	AB095903	—
	30987†	Queenstown, New Zealand	AB096052	AB095904	—
	45843†	Hampshire, Massachusetts, USA	AB080788	AB095884	—
	45785†	Hampshire, Massachusetts, USA	AB080789	AB095885	—
	45840†	Lawrence, Massachusetts, USA	AB080791	AB095887	—
	45820†	Bronx, New York, USA	AB080790	AB095886	—
	45863†	Mendocino, California, USA	AB080787	AB095883	—
<i>A. m. var. alba</i>	GAL14284	Denali National Park, Alaska, USA	DQ060895	DQ060915	DQ060875
	GAL15453	North Pole, Alaska, USA	DQ060899	DQ060919	DQ060879
	GAL16735	Fairbanks, Alaska, USA	DQ060896	DQ060916	DQ060876
	49100†	Cascade, Idaho, USA	AB080793	AB095889	—
<i>A. m. var. formosa</i>	GAL4247	Juneau, Alaska, USA	DQ060894	DQ060914	DQ060874
	GAL15330	Fairbanks, Alaska, USA	DQ060891	DQ060911	DQ060871
	GAL15461	North Pole, Alaska, USA	DQ060900	DQ060920	DQ060880
	GAL16775	Fairbanks, Alaska, USA	DQ060892	DQ060912	DQ060872
	45883†	Piscataquis, Massachusetts, USA	AB080792	AB095888	—
	45060†	Amador, California, USA	AB080795	AB095891	—
	44761†	Alpine, California, USA	AB080794	AB095890	—
<i>A. m. var. regalis</i>	GAL2810	Dalton Highway, mile 122, Alaska, USA	DQ060904	—	DQ060884
	GAL3169	Eagle Summit, Alaska, USA	DQ060905	—	DQ060885
	GAL3688	Juneau, Alaska, USA	DQ060906	—	DQ060886
	GAL5505	Denali National Park, Alaska, USA	DQ060908	DQ060922	DQ060888
	GAL6027	Nome, Alaska, USA	DQ060909	—	DQ060889
	GAL16654	Fairbanks, Alaska, USA	DQ060907	DQ060921	DQ060887
	506†	Dovre, Oppland, Norway	AB080780	AB095855	—
	1539†	Gjøvik, Oppland, Norway	AB080781	AB095856	—
	4220†	Nordre-land, Oppland, Norway	AB080782	AB095857	—

*Sequences of isolates marked by † were published by Oda *et al.* (2004).

The entire ITS and partial LSU regions were PCR amplified in reaction mixtures containing 1.75 µL Ultrapure Water (Invitrogen), 1 µL 10 × Herculanase PCR buffer (Stratagene), 0.05 µL 100 mM dNTP mixture, 25 mM of each dNTP

(Applied Biosystems), 0.2 µL Herculanase DNA polymerase (Stratagene), 2 µL of 1 µM forward primer, ITS1F (Gardes & Bruns 1993) and reverse primer, TW13 (White *et al.* 1990), and 3 µL of template DNA at a concentration of

0.1 ng/ μ L. PCRs were performed using the following temperature programme for the two ribosomal gene regions: 95 °C/2 min, 34 cycles of 95 °C/0.5 min, 54 °C/1 min, 72 °C/2 min; and 72 °C/10 min. The concentration of the amplification products was determined using Picogreen (Molecular Probes). The amplification products were normalized to a concentration of 4 ng/ μ L and sequenced using the ABI BigDye version 3.1 Terminator Kit and an ABI 3730xl automated capillary DNA sequencer (Applied Biosystems). Because the amplification products were 1300+ bp long, we used two internal primers for cycle sequencing, ITS4 and CTB6 (White *et al.* 1990), in addition to the primers used in the PCRs.

Phylogenetic analysis

Sequence data obtained for both strands of each locus were edited and assembled for each isolate using CODONCODE ALIGNER version 1.3.4 (LI-COR). Sequence alignments were initiated using CLUSTAL W (Thompson *et al.* 1997) and subsequently corrected manually. Although none of the three loci contained ambiguously aligned positions, a hypervariable region was observed in the beta-tubulin data set corresponding to positions 60–86. These positions could still be aligned across all groups, yet there were a large number of gaps corresponding to a 21-bp deletion and several smaller indels. We recoded this region using INAASE 2.3b (Lutzoni *et al.* 2000) to retain the phylogenetic information present in the region without overweighing the deletions. The code matrix was attached to the alignment and was included in maximum-parsimony (MP) analyses. Analyses were conducted in multiple steps using the MP method in PAUP* 4b10 (Swofford 2002), and Bayesian analysis in MRBAYES 3.0 (Huelsenbeck & Ronquist 2001). Because the methods above follow different theories and algorithms, only congruent branching patterns found in both types of analyses were considered meaningful. To test the combinability of DNA sequence data from different loci, the partition homogeneity test (PHT) was performed on only parsimony-informative sites with 1000 randomized data sets, using heuristic searches with simple addition of sequences. The best-fit evolutionary model for Bayesian analyses was determined for each data set by comparing different evolutionary models with varying values of base frequencies, substitution types, α -parameter of the γ -distribution of variable sites, and proportion of invariable sites via the Akaike information criterion (AIC) using PAUP* and MODELTEST 3.06 (Posada & Crandall 1998). MP analyses were carried out with the heuristic search option using the 'tree-bisection-reconnection' (TBR) algorithm with 100 random sequence additions to find the global optimum with MAXTREES set to 10 000 in the combined analyses. To test the stability of clades detected, the bootstrap test (Felsenstein 1985) was used with 'full heuristic search'. The

number of replicates were 1000 and 100 for the individual and combined data sets, respectively. In Bayesian phylogenetic analyses, 200 000 generations were run in four chains for the single-locus, and 1 000 000 generations for the combined data sets. The chains were sampled every 100th generation. When the likelihood scores of trees sampled approached similar values, they were considered to have converged. In each run, trees after this convergence point were used to compute a majority rule consensus tree. Gaps were scored as 'new state' in MP and as 'missing data' in Bayesian analyses. To compare the likelihood of different tree topologies, two-tailed Kishino–Hasegawa tests were used (Kishino & Hasegawa 1989) with parsimony and likelihood settings specified beforehand.

Supertree construction

We constructed supertrees using the Matrix Representation with Parsimony method (MRP) (Baum 1992; Ragan 1992), a supertree approach for analysing and combining individual trees derived from multiple data sets. One of the biggest advantages of using supertree methods is the ability to combine phylogenetic information present in only partially overlapping data sets (i.e. the ability to overcome missing data). In MRP, the topology of each source tree is recoded as a series of binary characters describing each node. Each character describes a clade in a tree such that descendants of the node are scored as '1', all others as '0' except for missing data that is scored '?'. The resulting matrix is then analysed using parsimony to produce a consensus estimate based on the source trees (Jones *et al.* 2002). MRP handles conflict by weighing the evidence in different source trees without any tree having the power of veto (Creevey & McInerney 2004).

While published supertree analyses have generally been based on pre-existing phylogenies as source trees, we used the Bayesian trees generated earlier in this study for individual loci to construct supertrees. Bininda-Emonds & Sanderson (2001) assessed the accuracy of MRP and concluded that weighted MRP performed at least equally well or better than the total evidence approach (analyses of combined original data sets), and always better than nonweighted MRP. They recommended weighting source trees based on node support, such as bootstrap values, whenever possible. Following this path, but adopting a slightly different approach, we chose 100 random trees for each locus from the sets of trees generated in Bayesian analyses after the convergence of likelihood scores. This enabled us to weight the nodes according to their posterior probability values (i.e. their observed frequencies in the sampled trees). We produced the MRP matrix by combining the matrix representation of all 300 trees in PAUP. MP analyses were carried out with the heuristic search option using the TBR algorithm with 100 random sequence additions. The stability of clades was

evaluated by bootstrap test, resampling nodes as characters, used with 'full heuristic search', and 1000 replicates.

Phylogeographic analyses

Phylogeographic patterns linked to the different phylogenetic species and the species complex as a whole were investigated using NCA (Templeton 1998). To improve the performance of the NCA, we removed the haplotype representing the sample from New Zealand. Because *A. muscaria* is not native to the Southern Hemisphere, including this isolate would have introduced an unnecessary source of error in the process of inferring the phylogeographic history of the species complex. Maximum-parsimony haplotype networks were generated by TCS version 1.18 (Clement *et al.* 2000) and were used to define a series of nested clades that in turn were used to perform random, two-way contingency permutation analysis to detect any association between geographic distribution and genetic variation (Templeton 1998). The nested clade information, sample size for each haplotype, and geographic location of each clade (latitude and longitude coordinates) were entered into the software package GEODIS version 2.0 (Posada *et al.* 2000). GEODIS was used to calculate clade distance (D_c) and nested clade distance (D_n), and to test them for significance at $\alpha = 0.05$ level using a permutation technique with 10 000 resampling replicates (Posada *et al.* 2000). D_c was calculated as the average distance of all individuals in clade 'X' from the geographic centre of that clade, while D_n was the average distance of individuals in clade 'X' from the geographic centre of clades of the next highest nesting level. Where significant D_c and/or D_n values were detected, a set of criteria was used to detect the effects of contemporary (e.g. gene flow) vs. historical (e.g. allopatric fragmentation, and range expansion) processes (Templeton 1998; Posada *et al.* 2000). In addition, nucleotide diversity (π , the average pairwise nucleotide differences per site) was calculated using ARLEQUIN version 2.0 (Schneider *et al.* 2000) to compare the amount of genetic diversity found in Alaska to that of other geographic groups.

Coalescent analyses

Identical sequences were collapsed into haplotypes using SNAP MAP (Aylor & Carbone 2003) and SITES version 1.1 (Hey & Wakeley 1997), excluding insertion or deletions (indels) and categorizing base substitutions as phylogenetically uninformative or informative, and transitions vs. transversions. Although coalescent methods can take full advantage of the data, they make strict assumptions, such as neutrality and lack of recombination, that must be verified a priori. Tajima's D (Tajima 1989) and Fu and Li's D^* and F^* (Fu & Li 1993) test statistics were calculated with DNASP version 3.53 (Rozas & Rozas 1999) to test for

departures from neutrality. SNAP Clade and SNAP Matrix (Markwordt *et al.* 2003) were used to generate site compatibility matrices to detect recombination blocks. Based on the evidence for geographic population structure as detected by NCA, MDIV (Nielsen & Wakeley 2001) was used to distinguish equilibrium migration vs. shared ancestral polymorphisms between subdivided populations. MDIV applies Markov chain Monte Carlo (MCMC) coalescent simulations to estimate the population mean mutation rate, divergence time, migration rate, and the time since the most recent common ancestor (TMRCA). Subsequently, we reconstructed the genealogy with the highest root probability, the ages of mutations, and the TMRCA of the sample using coalescent simulations with population subdivision in GENETREE version 9.0 (Griffiths & Tavaré 1994).

Molecular clock analyses

To estimate the ages of the nodes, maximum-likelihood (ML) analyses were conducted using PAUP* 4b10 based on LSU sequences, with and without the enforcement of a molecular clock. The data set contained the same taxa with eight additional sequences representing other groups of Basidiomycota (*Ustilago maydis* AF453938, *Auricularia delicata* AF291290, *Boletus pallidus* AF457409, *Stropharia coronilla* AF059232, and *Melanophyllum haematospermum* AF261476). The likelihood values of the resulting trees were compared by the χ^2 -test at $\alpha = 0.05$ significance level. The test statistic was equal to twice the difference of log-likelihood scores, which is χ^2 distributed with $n - 2$ degrees of freedom, where n is the number of terminal taxa (Page & Holmes 1998). Absolute ages of nodes were estimated by fixing the age of the *Ustilaginomycetes*/*Hymenomycetes* separation at 430 million years ago (Ma) (based on Berbee & Taylor 2001). The branch length and standard error values were estimated using PAML (Yang 1997).

Results

Phylogenetic analyses

The ITS, beta-tubulin, LSU and the combined data sets consisted of 717, 468, 625, and 1810 characters, respectively, including gaps. There were 36, 14, 12, and 62 parsimony-informative characters, respectively. The Tamura-Nei model (Tamura & Nei 1993), with calculated proportion of invariable sites and equal variation rates for all sites (TrN + I), was selected as the best-fit evolutionary model for all three individual data sets.

In Bayesian analysis of the ITS, beta-tubulin, LSU, and combined data sets, the consensus trees were computed from 1162, 484, 1510, and 5238 trees, after discarding the first 839, 1517, 491, and 4763 trees as 'burn-in', respectively. MP analyses generated 16, 39, 3, and 10 000 equally parsimonious

trees for the ITS, beta-tubulin, LSU, and combined data sets, respectively. The ITS phylograms were 95 steps long with consistency index (CI) = 0.874, retention index (RI) = 0.965, rescaled consistency index (RC) = 0.843, and homoplasy index (HI) = 0.126. Trees generated from the beta-tubulin alignment had the following scores: length = 109 steps, CI = 0.862, RI = 0.840, RC = 0.725, and HI = 0.138. The LSU phylograms were 22 steps long, and had scores of CI = 0.864, RI = 0.906, RC = 0.783, and HI = 0.136. MP trees of the combined data set were 231 steps long with CI = 0.848, RI = 0.925, RC = 0.785, and HI = 0.152.

Three major clades receiving high support (Clades I–III, Fig. 1) were detected within *Amanita muscaria* based on phylogenetic analyses of the ITS and LSU alignments; however, the relationships among Clades I, II, and III were not clear. Although both Clades I and III formed monophyletic groups, only Clade I was well supported in the beta-tubulin phylograms, despite a moderate number of parsimony-informative sites. All three groups had unique 'signature sequences' in the hypervariable region corresponding to positions 60–86 in the alignment. In this region, isolates in Clades I and III were monomorphic within their clades and characterized by a 21-bp deletion in Clade I, and several small indels in Clade III. Although many isolates of Clade II were polymorphic, they all shared a GT (positions 82–83) 'insertion' unique to the clade, and none of them had sequences identical to the two other groups. (This 'insertion' should be interpreted as nucleotides that are missing in both Clades I and III, and does not refer to the evolutionary history of the sites.) While the beta-tubulin MP tree did not support the monophyly of Clade II, it did not show significant conflict with the ITS and LSU trees. When Clades I, II, and III were under monophyletic constraint, the equally parsimonious trees (length = 111, CI = 0.847, RI = 0.819, RC = 0.694, and HI = 0.153) were only two steps longer than the unconstrained trees described earlier. The Kishino–Hasegawa test revealed that the difference between the two topologies was not significant ($P = 0.48$). Apparently, this lack of conflict was not due to low phylogenetic signal in beta-tubulin. A permutation tail probability (PTP) test (Archie 1989; Faith & Cranston 1991) revealed that the beta-tubulin locus contributes phylogenetic signal to the combined data set, because tree length of the original beta-tubulin phylogram was significantly shorter ($P < 0.01$) than the length of the trees generated based on randomly permuted beta-tubulin data sets. As expected, Clades I, II, and III were strongly supported in analyses of the combined data set with 96%, 99%, and 100% MPB and all 1.0 BPP values, respectively (Fig. 1A). A southeast Alaskan subclade (II/A) also received high support: 96% MPB and 1.0 BPP. Phylogenetic relationships among Clades I, II, and III remained unclear, as none of the groupings were supported by significant MPB and BPP values.

Supertree construction

Matrix representations of the ITS, beta-tubulin, and LSU resulted in 93, 73, and 39 characters (recoded nodes), respectively, for each tree. Therefore, the entire data set containing matrix representations of 100 trees for each locus contained 20 500 characters. Out of these, 9800 characters were parsimony-informative. The single most parsimonious tree (see Supplementary material) was 33 803 steps long with CI = 0.606, RI = 0.743, RC = 0.451, and HI = 0.394. All of the major clades described earlier and subclade II/A were well resolved.

Evolution of morphological varieties

Representatives of multiple, morphologically distinct varieties were found in several clades. To test whether specimens with shared phenotype were monophyletic, Kishino–Hasegawa tests were performed. Tree length and likelihood score of the most likely of the 10 000 MP trees constructed from the unconstrained combined data set were compared to the length and likelihood scores of the most likely MP tree under the constraint of monophyly of the morphological variety in question. Separate analyses were conducted for each morphological variety to detect whether any one of the three *A. muscaria* varieties (var. *alba*, var. *formosa*, and var. *regalis*) was monophyletic. Other varieties were not tested, because for many isolates only the species identity was known, without reference to the variety, making it impossible to distinguish between the two varieties with red pileus: *A. muscaria* var. *muscaria* (often referred to only by species name) and *A. muscaria* var. *flavivolvata*. In all analyses, the constrained trees were significantly worse (i.e. had significantly more steps and lower likelihood scores) than the unconstrained trees (all $P < 0.01$) (Table 2).

Phylogeographic analyses

A total of 25 haplotypes were detected in *A. muscaria* isolates from the Northern Hemisphere (Fig. 2). Although these haplotypes grouped in three separate networks at 95% connection limit, representing the major clades described earlier, it was possible to connect these clades at 92% connection limit. The nested haplotype networks of Clades I, II, and III are shown in Fig. 2. Haplotypes XII, I, and XXIII were inferred to have the highest outgroup probability in the separate cladograms representing Clades I, II, and III, respectively. In the total cladogram connecting all clades, haplotype I had the highest outgroup probability. The missing intermediate haplotypes were retained during the nesting procedure for consistency in nesting (Crandall 1996).

In the network of Clade I, the null hypothesis of no association between genotype and geographic origin was

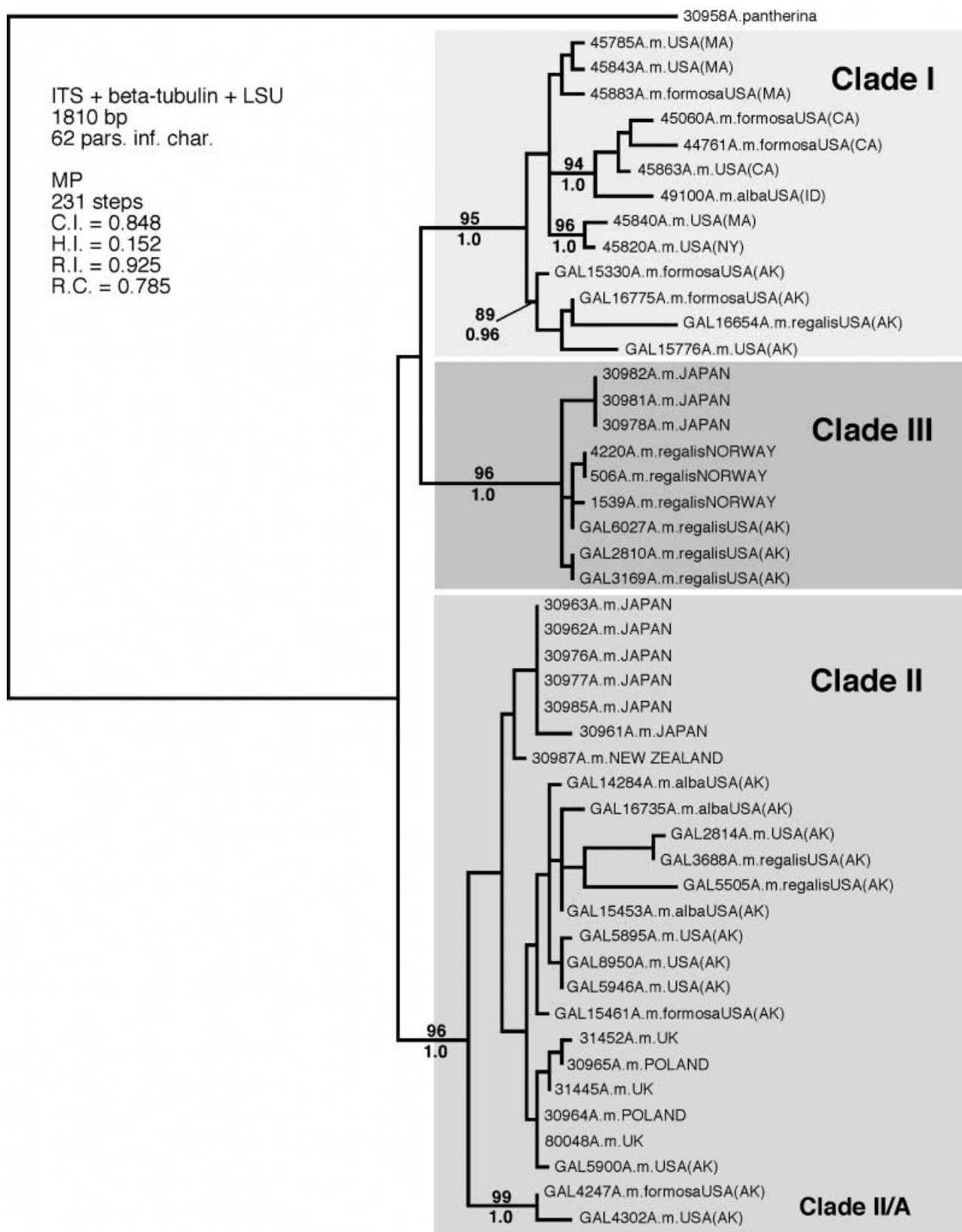
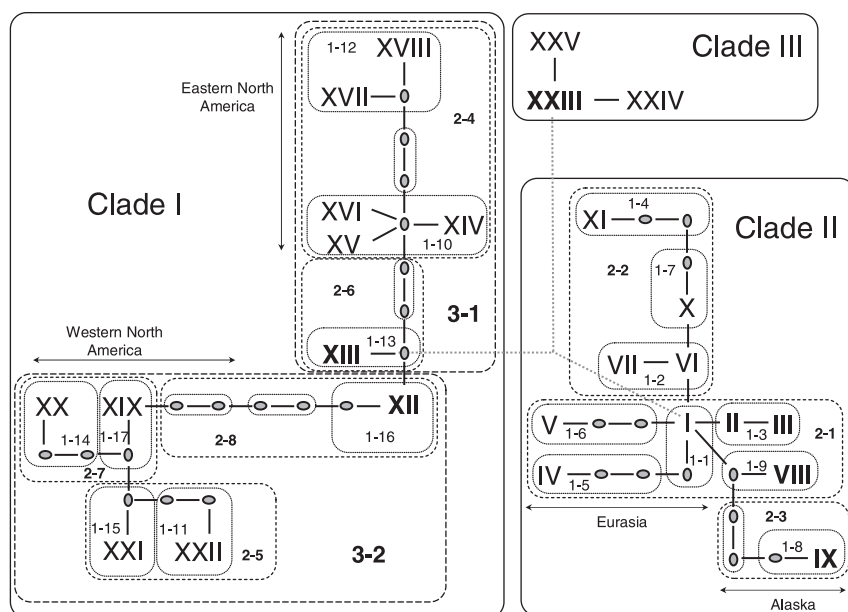


Fig. 1 One of the 10 000 equally parsimonious trees for the combined data set with >70% maximum-parsimony bootstrap and >0.95 Bayesian posterior probability values shown above and below the supported branches, respectively.

Table 2 Results of Kishino–Hasegawa tests for monophyly of morphological varieties based on maximum-parsimony analyses of the combined data set

Morphological variety	Tree	–ln L	Diff. –ln L	<i>P</i>	No. of steps	Diff. no. of steps	SD	<i>t</i>	<i>P</i>
	unconstrained	3476.1312	Best		212	Best			
<i>A. m. var. alba</i>	constrained	3572.4051	96.1739	0.002	237	25	6.38	3.92	< 0.001
<i>A. m. var. formosa</i>	constrained	3656.3632	180.1320	< 0.001	248	36	7.16	5.03	< 0.001
<i>A. m. var. regalis</i>	constrained	3624.9986	148.7674	< 0.001	240	28	6.45	4.34	< 0.001

**Fig. 2** Maximum-parsimony haplotype network constructed based on ITS sequences at 95% connection limit. Gaps were scored as 'new state'. Roman numerals indicate sampled haplotypes, while grey ovals represent unsampled extant or extinct haplotypes. Dotted grey lines indicate connections that were only found at connection limit $\leq 92\%$. Haplotypes in bold have been found in Alaska.

rejected ($P < 0.05$) in clade 3-2 and the total cladogram, with significant, large interior clade (I) and interior vs. tip clades (I–T) D_n values in clade 3-2, and with significant, small tip (T) and I D_c values and small I–T D_n value in the total cladogram (Table 3). Based on the most up-to-date (14 July 2004; <http://darwin.uvigo.es/software/geodis.html>) version of the inference key of Templeton (1998), the significant statistical association between haplotype and geography was due to contiguous range expansion (CRE) in the total network of Clade I. There was insufficient information to differentiate between CRE, long-distance colonization (LCD), and past fragmentation (PF) in clade 3-2 (Table 3).

In the network of Clade II, a statistically significant association was found between genotype and geographic origin in clades 2-1 and 2-2. Significant, large I D_n and I–T D_n , and significant, small T D_c and T D_n values were found in clade 2-1, while significant, small I D_c , I D_n , and I–T D_n values were detected in clade 2-2. Although we were not able to differentiate between allopatric fragmentation (AF) and isolation by distance (IBD) in clade 2-1, CRE was inferred in clade 2-2. Also, we detected significant

genotype–geography association in the total Clade II cladogram with CRE as the underlying mechanism.

The Clade III network contained only a single one-step clade in which significant, large I and I–T D_n values were detected. However, it was not possible to discriminate between IBD and AF due to the small number of sampled haplotypes.

NCAs of the total cladogram containing Clades I, II, and III detected significant, small T D_c and T D_n , and significant, large I–T D_c and I–T D_n values. The inference that Clade II was the interior clade was justified by the rcs program, which designated haplotype I in Clade II to have the highest outgroup probability that correlates with haplotype age. Allopatric fragmentation was inferred to explain the ancient divergence of *A. muscaria* populations (Table 3). This hypothesis is further supported by the presence of long branches separating the major clades.

Coalescent analyses

After removing the indels, seven previously detected haplotypes collapsed, resulting in a total of 18 distinct ITS haplotypes (Table 4). The site compatibility matrix showed

Haplotype			1-step clade			2-step clade			3-step clade			Species-level clade		
Name	D _c	D _n	Name	D _c	D _n	Name	D _c	D _n	Name	D _c	D _n	Name	D _c	D _n
I	0	0	1-1(I)	0	6974 ^L									
II(I)	561	555												
III(T)	0	225												
I-T	561	331	1-3(T)	532 ^S	1397 ^S									
IV	0	0	1-5(T)	0	6974									
V	0	0	1-6(T)	0	6974									
VIII	0	0	1-9(T)	0	2052									
			I-T	−409	4668 ^L									
			1-2-3-4-9-10 No: AF/IBD			2-1(I)	2458 ^S	4724 ^S						
VI(I)	2226	2067												
VII(T)	0	1186												
I-T	2226	880	1-2(I)	1937 ^S	3773 ^S									
XI	0	0	1-4(T)	0	6175									
X	0	0	1-7(T)	0	6175									
			I-T	1937	−2403 ^S									
			1-2-11-12 No: CRE			2-2(T)	4374	7468 ^L						
IX	0	0	1-8	0	0	2-3(T)	0	4310 ^S						
						I-T	−1186	−2218 ^S						
						1-2-11-12 No: CRE						Clade II(I)	5342	5630
XIII	0	0	1-13	0	0	2-6(I)	0	4610						
XIV	0	0												
XV	0	0												
XVI	0	0	1-10(I)	0	123									
XVII(I)	0	49												
XVIII(T)	0	196												
I-T	0	147	1-12(T)	79	123									
			I-T	−79	−1	2-4(T)	123	748						
						I-T	−123	3861	3-1(T)	1458 ^S	1706			
XII	0	0	1-16	0	0	2-8(I)	0 ^S	2384 ^L						
XIX	0	0	1-17(I)	0	795									
XX	0	0	1-14(T)	0	258									
			I-T	0	537	2-7(T)	392	1119						
XXI	0	0	1-15(I)	0	0									
XXII	0	0	1-11(T)	0	0	2-5(T)	0	1092						
						I-T	−196	1278 ^L						
						1-2-11-12-13-14 Yes:CRE/LDC/PF			3-2(I)	1177 ^S	2735			
									I-T	280	−1028 ^S			
									1-2-11-12 No: CRE			Clade I(T)	2077 ^S	2765 ^S
XXIII(I)	6136	6287 ^L												
XXIV(T)	0	2783												
XXV(T)	0	2783												
I-T	6136	3504 ^L												
1-2-11-17-4-9-10 No: AF/IBD												Clade III(T)	4203	5738
												I-T	2395 ^L	1649 ^L
												1-2-3-4-9 No: AF		

Table 4 Polymorphic sites in the ITS haplotypes collapsed after removing indels from the original ITS data set for the subsequent coalescent analyses. Position refers to that in the original alignment, site number is the designation of the given mutation as shown on Fig. 3, site type refers to either transition (t) or transversion (v) change with regard to the consensus sequence. Roman numbers refer to haplotype designations on Figs 2 and 3. Haplotypes marked with asterisk include more than one haplotypes from the nested clade analyses, where indels were not excluded

Position	11111222223344444555666
	68995678901247868122367029233
	72899049848224345327377164447
Site number	1111111111222222222
	12345678901234567890123456789
Site type	ttttvtttvtvtvtttvtvtvtvtvt
Character type	-iii-iii-i-i-----iii--i-i---
Consensus	CGCTTGCTGCACAACGTTGAAGTGCGCCT
Haploypst†	
I*	.ATC...C.A.A.....G..T.T...
III	.ATC...CAA.A.....G..T.T...
X	.ATC...C.A.AT.....G..T.T...
VII	.ATC...C.A.A...A...G..T.T...
XI	.ATC...C.A.A....A..G..T.T...
IX	...C...C.A.A.....G..T.T.T.
XXIII	...C.AT...A.....
XXIV	...C.AT...A..T.....
XXV	...C.AT...A.....T.....
XIV	C...C.....
XV	...A.....C.....
XX	G..G..C.C.....
XVII	T.....CC.....
XVIII	CC.....
XXI*	C..T.....
XVI	C.....T..
XIX	C.....
XII*	C.....C

†Collapsed haplotypes after removing indels: I* = I, II, IV, V, VI, VIII; XXI* = XXI, XXII; XII* = XII, XIII.

conflict at positions 123–124; therefore these were excluded from subsequent analyses. The coalescent-based ITS genealogy was informative for inferring the mutational history with respect to variation between and within the major clades (Fig. 3). It also confirmed that Clade III likely is the sister clade of Clade I, with a divergence time estimate of 0.939, measured in coalescent units of $2N$, where N is the population size. The mean ages of the first radiation of Clades I, II, and III are 0.128, 0.276, and 0.507, respectively. This suggests that the oldest within-clade radiation may have taken place in Clade III, despite the low number of observed mutations, and that mutation rate in Clade III is much lower than mutation rates observed in the two other clades. Also, Clade I seems the youngest, suggesting that the range expansion in North America likely started more recently than that in Eurasia.

Molecular clock analyses

ML analyses of the LSU data set conducted with and without the enforcement of a molecular clock resulted in one tree each with likelihood values of $-\ln L_{\text{clock}} = 2124.1808$ and $-\ln L_{\text{no clock}} = 2122.2267$, respectively. Since twice the difference of likelihood scores ($2 \times 1.9541 = 3.9082$) was smaller than the critical $\chi^2_{\alpha=0.05; d.f.=8} = 15.51$ value, the difference between the trees obtained with and without enforcing the molecular clock is not significant. The age of the first separation within *A. muscaria* (between Clades I and II) was estimated at 7.48 ± 4.53 Ma.

Discussion

Phylogenies inferred from the individual and combined data sets, and the supertree concordantly suggested three distinct clades in the *Amanita muscaria* species complex. These clades were first detected by Oda *et al.* (2004) and were referred to as geographic groups (i.e. allopatric populations). However, our data suggest that these groups are not entirely allopatric, but have geographic ranges that overlap in Alaska. We found representatives of all three clades in interior Alaska, and specimens from Clades II and III in western arctic Alaska. Because the nonconflicting gene genealogies indicate the lack of gene flow among the clades, we conclude that these groups represent distinct phylogenetic species with sympatric populations in Alaska (Fig. 4A).

Interestingly, all detected phylogenetic species within *A. muscaria* share at least two morphological varieties with other species. Clades I and II both contain at least four (var. *alba*, var. *formosa*, var. *regalis*, var. *muscaria* and/or var. *flavivolvata*), while at least two (var. *regalis*, var. *muscaria* and/or var. *flavivolvata*) have been found to date in Clade III. The most parsimonious explanation for the evolution of these morphological varieties is the presence of ancestral polymorphism in pileus and wart colour that pre-dated the separation of the phylogenetic species. In addition, the pileus colour may be influenced by unknown biotic or abiotic environmental factors. Although different colour varieties generally were found in all sampled climatic zones (temperate, boreal, and arctic-subalpine), eight of the nine *A. muscaria* var. *regalis* specimens were from regions with cold climate (either boreal, arctic or subalpine). The only *A. muscaria* var. *regalis* found in a more temperate climate was the one from the rainforests of southeastern Alaska, only a few miles from the subalpine zone. This finding confirms its rather limited distribution that is restricted to coniferous forests, low arctic and subalpine regions of northern and central Europe, and Alaska (Miller 1982; Jenkins 1986).

It is a widely held assumption that low genetic variation is indicative of recent colonization and that the greatest

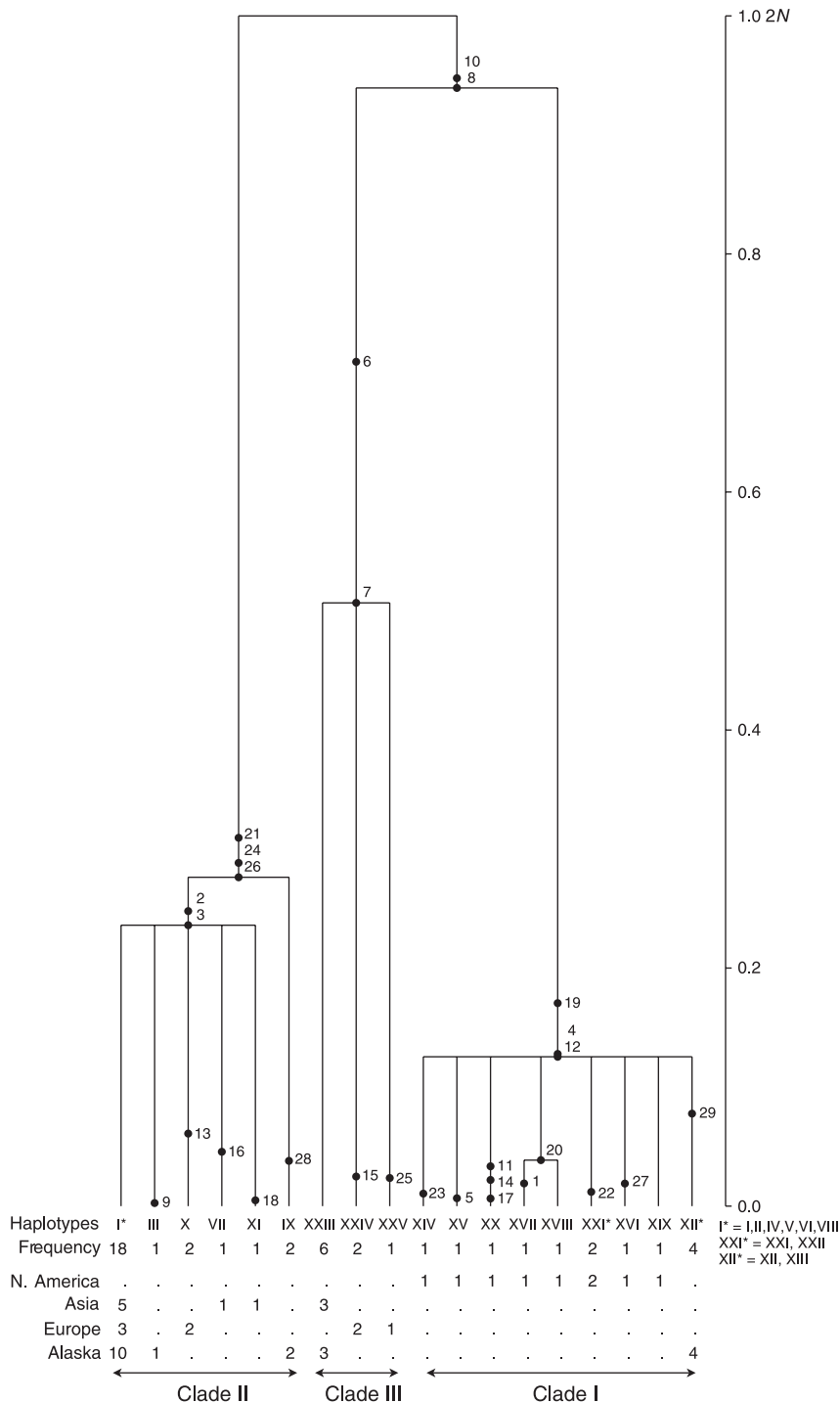


Fig. 3 Coalescent-based genealogy with the highest root probability ($L = 6.4693 \times 10^{-54}$, $SD = 6.3319 \times 10^{-51}$) showing the distribution of mutations for the ITS region in the major clades. The inferred genealogy is based on 2 million simulations of the coalescent with a Watterson's estimate of $\theta = 4.0$. The timescale is in coalescent units of $2N$, where N is the population size. Mutations and bifurcations are time ordered from the top (past) to the bottom (present). Mutation designations correspond to the site numbers in Table 4. The numbers below the tree designate the distinct haplotypes, their observed frequencies in total and in the different geographic regions.

genetic diversity should be found among isolates from regions that have been inhabited for the longest period. Oda *et al.* (2004) hypothesized that the ancestral group of *A. muscaria* evolved in Eurasia and migrated to North America via land bridges. In our sample, we found the greatest genetic diversity in Alaskan populations ($\pi = 0.013094 \pm 0.00702$, $n = 20$ specimens), followed by Eurasia ($\pi = 0.011446 \pm 0.006216$, $n = 18$), and by North America ($\pi =$

0.009614 ± 0.005676 , $n = 9$). High genetic diversity in Beringia has also been reported in surveys of populations of the Columbian ground squirrel, *Spermophilus columbianus* (MacNeil & Strobeck 1987), the swallowtail butterfly, *Papilio machaon* (Sperling & Harrison 1994), and the ground beetle *Amara alpina* (Reiss *et al.* 1999).

The nucleotide diversity estimates and the results of the phylogenetic, phylogeographic, and coalescent analyses

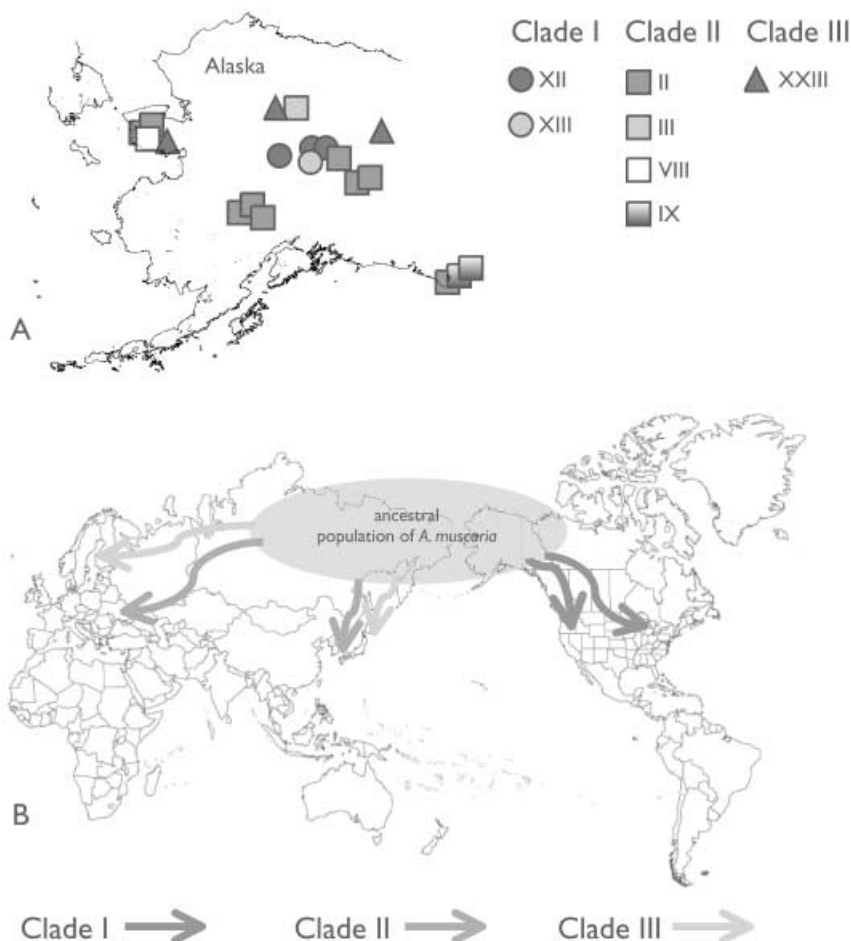


Fig. 4 (A) Outline map of Alaska showing the geographic distribution of the sampled haplotypes of the three phylogenetic species. (B) Mercator world map showing the putative ancestral population and possible migration routes of the phylogenetic species.

concordantly suggest that the centre of origin of *A. muscaria* likely is in Beringia (Fig. 4B). We hypothesize that the ancestral *A. muscaria* population evolved in the humid, temperate forests that covered much of Beringia in the late Tertiary (Hultén 1968; Graham 1999). Although it is difficult to estimate the divergence times of the major clades due to the wide range of time estimates of our molecular clock analyses (7.48 ± 4.53 Ma), the fragmentation of the ancestral population into at least two major clades might have taken place as a consequence of the opening of the Bering Strait about 12 Ma. Clade III likely is a sister group of Clade I, as inferred from the phylogenetic and coalescent analyses, and it is safe to conclude that the ancestral population was divided into Eurasian and Alaskan populations. With the cooling climate, some populations of Clades I and II likely migrated southward in North America and Eurasia (Fig. 3B), respectively, as is supported by the contiguous range expansion inferred in both clades by NCA. However, coalescent mutation age estimates suggest that the radiation and southward expansion may have happened more recently in North America (Clade I) than in Eurasia (Clades II and III).

In North America, the expansion of Clade I took two main directions: (i) southward along the western side the Rocky Mountains which resulted in the extant populations in the western United States, represented by samples from California and Idaho; and (ii) southeastward along the eastern slopes of the Rocky Mountains which allowed the establishment of populations in the eastern United States, represented by samples from Massachusetts and New York. This latter route was shared by numerous plant taxa that originated in Alaska and replaced many species along their migration to the southeast (Budantsev 1992). Interestingly, we did not find any haplotype in Alaska that descended from other North American haplotypes. This suggests that populations of *A. muscaria* survived the glacial maxima in Alaskan refugia and there was no significant postglacial migration from southern populations back to Alaska. On the contrary, Alaskan populations likely gave rise to both eastern and western North American lineages before the Quaternary period.

Range expansion patterns in Clades II and III are more difficult to interpret, partly because of large unsampled areas in Asia. The NCA results in clade 2-1, which is the

interior clade and the only one containing both Alaskan and Eurasian samples, indicate allopatric fragmentation or isolation by distance. Isolates from unsampled areas in Asia are needed to clarify this question. However, a more basal bifurcation, separating the southeast Alaskan group (II/A, ITS haplotype IX) from the rest of Clade II, can be observed in the combined phylogeny and the coalescent-based genealogy. It is somewhat surprising that no evidence was found for migrations of *A. muscaria* from Eurasia to North America/Alaska, despite what had been suggested by Oda *et al.* (2004). This question should be addressed by further phylogeographic studies with increased sample size.

Beside the southward range expansions detailed above, populations of all three species clades have continuously inhabited Beringia. In the Quaternary, the Illinoian and Wisconsinian glaciations likely restricted *A. muscaria* to isolated refugia of boreal forest and shrub tundra along the Yukon and Tanana rivers in interior Alaska that remained unglaciated (Hultén 1968; Graham 1999). While it is unclear whether conifers were present in the region at glacial maxima, it is very likely that *Betula*, *Dryas*, *Populus* and *Salix* inhabited at least some parts of the region (Edwards *et al.* 2000; Swanson 2003) and likely were able to maintain refugia of *A. muscaria*. The ecological plasticity of *A. muscaria*, i.e. the broad range of potential mycorrhizal hosts, including *Betula*, *Dryas* and *Salix* spp. in subalpine tundra (Miller 1982), supports the hypothesis of glacial refugia in Alaska. In addition, although earlier pollen data did not indicate the presence of *Picea* in Beringia at the last glacial maximum (Edwards *et al.* 2000; Swanson 2003), recent pollen data (Brubaker *et al.* 2005) and phylogeographic analyses based on DNA sequences (F.S. Hu, personal communication) suggest the existence of glacial refugia of *P. glauca* and *P. mariana* in eastern Beringia.

In this study, we documented the existence of three distinct phylogenetic species in the *A. muscaria* species complex. Furthermore, we hypothesized evolutionary and phylogeographic processes leading to speciation and intraspecific population structures. Future studies should include specimens from unsampled regions to further elucidate the phylogeography of the species complex. Among these, Siberia is of particular interest, because it might possess genetically diverse populations, including putatively ancestral Beringian elements.

The implications of our results are not restricted to *A. muscaria*. The phylogeographic patterns seen here might be shared, at least in part, by many boreal ECM fungi in the Northern Hemisphere, particularly in North America. It is certain that many plant lineages contributing to the recent boreal and temperate flora evolved within high-latitude forests of Beringia during the Tertiary and migrated southward as the climate cooled (Graham 1999). Furthermore, because there is increasing evidence for boreal forest glacial refugia in Alaska, Holocene migrations of boreal plants

and ECM fungi likely occurred not only northward from southern refugia, but southeastward from Alaskan refugia. This is supported by the rapid postglacial colonization of the present boreal regions by *Picea*, and the fact that no recent migration of *A. muscaria* from more southern regions of North America to Alaska was detected in our analyses. As a consequence, we propose that Beringia is not only the original and longest inhabited region for many plant and animal taxa, but may represent a biodiversity 'hotspot' for high-latitude ECM fungi as well.

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Supplementary material

The supplementary material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2799/MEC2799sm.htm>

Fig. S1 Supertree constructed by the matrix representation with parsimony method.

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Dr. Geml is interested in the systematics, evolution and biogeography of ectomycorrhizal Basidiomycota, particularly in Beringia. Dr. Laursen has carried out a 37-year study of high latitude fungal taxonomy, morphology and ecological relationships of the higher fungi within extreme environments. He has developed an herbarium of approximately 18,900 fungal, lichen and moss collections, creating a valuable genomic resource for further studies. The Taylor lab seeks to understand the ecological and evolutionary dynamics of plant-fungal interactions, with emphases on ectomycorrhizae and orchid mycorrhizae, and also uses metagenomics approaches to better understand the diversity and function of boreal forest fungi.



Short communication

Amanita muscaria and *Amanita pantherina* poisoning: Two syndromes

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ABSTRACT

Amanita muscaria contains more excitatory ibotenic acid and less depressant muscimol compared to *Amanita pantherina*. In this study *A. muscaria* poisoned patients were more often confused (26/32, $p = 0.01$) and agitated (20/32, $p = 0.03$) compared to those poisoned with *A. pantherina* (8/17 and 5/17). Patients poisoned with *A. pantherina* were more commonly comatose (5/17) compared to those poisoned with *A. muscaria* (2/32) ($p = 0.03$). In conclusion, the so-called ibotenic or pantherina-muscaria syndrome might be divided into two subtypes.

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1. Introduction

About 200 kinds of edible mushrooms are grown in Slovenia and around 50 of them are more or less poisonous (Kirbiš and Sinkovič, 2009). Mushroom poisoning in Slovenia is common, especially in fall, and is associated with traditional wild mushroom picking and cookery. The most severe and life-threatening poisonings from the ingestion of wild mushrooms are those with the *Amanita* species. *Amanita phalloides* (dead cap) is the cause of 90% of deaths due to mushroom poisoning as it contains the highly hepatotoxic and nephrotoxic peptide amatoxin (Kirbiš and Sinkovič, 2009; Li and Oberlies, 2005). On the other hand, *Amanita muscaria* (fly agaric) and *Amanita pantherina* (panther cap) poisoning with neurological symptoms due to the neurotoxic isoxazoles ibotenic acid

and muscimol are also common in Slovenia. Surprisingly, searching PubMed we have found only 6 case reports of *A. muscaria* (Brvar et al., 2006; Hohn and Schoenemann, 2000; Satora et al., 2005) or *A. pantherina* (Magdalan and Antonczyk, 2007; Satora et al., 2006) poisoning and two case series with 9 children (Benjamin, 1992) and 10 adults including 5 adult *A. muscaria* and 5 *A. pantherina* poisoned patients (Łukasik-Glebocka et al., 2011). Unfortunately, in the childrens' case series the clinical pictures were presented uncorrelated to the *Amanita* species (Benjamin, 1992).

After mushroom ingestion ibotenic acid and muscimol are rapidly absorbed from the gastrointestinal tract and can be detected in the urine within 1 h. Ibotenic acid and muscimol readily cross the blood–brain barrier via an active transport system (Olpe and Koella, 1978) and exert their effects primarily on the central nervous system, where they act as neurotransmitter agonists (Michelot and Melendez-Howell, 2003; Satora et al., 2005, 2006). Ibotenic acid acts as an excitatory amino acid at the glutamate receptors, while muscimol is a gamma-aminobutyric acid (GABA) receptor agonist and therefore has a depressant

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effect on the central nervous system (Krogsgaard-Larsen and Hansen, 1992; Michelot and Melendez-Howell, 2003). In addition excitatory ibotenic acid is spontaneously converted to the depressant muscimol by decarboxylation (Nielsen et al., 1985). The clinical picture of *A. muscaria* and *A. pantherina* poisoning involving the central nervous system and presenting with hallucinations, confusion, agitation, seizures and coma is described as ibotenic or pantherina-muscaria syndrome (Michelot and Melendez-Howell, 2003). However, this syndrome is very inconsistently described (Benjamin, 1992) and in the only adult case series *A. pantherina* poisoning resulted in coma in 4 out of 5 cases, but no coma was observed in *A. muscaria* poisoning (Łukasik-Głębicka et al., 2011). Nevertheless, the distinction of the clinical picture after ingestion of the two mushroom species was not further evaluated due to the small number of reported cases.

Accordingly, the aim of this study was to compare the clinical presentations in *A. muscaria* and *A. pantherina* poisoning.

2. Methods

The study was conducted in the 9-bed toxicology ward of the University Medical Centre Ljubljana Poison Control Centre, the primary city hospital for the Slovenian capital city of Ljubljana serving a population of 400,000 inhabitants and tertiary referral hospital centre serving a population of 2,000,000.

In this study we retrospectively included all patients admitted for *A. muscaria* and *A. pantherina* over a period of 33 years (1980–2013). The inclusion criteria were mycological confirmation of *A. muscaria* or *A. pantherina* ingestion based on macroscopic and/or microscopic mushroom analysis.

The medical files of enrolled patients were reviewed and the following data collected: age, sex, cause of poisoning and which edible mushroom was considered to be ingested, time of symptoms onset, clinical presentation and treatment. In the analysis of signs and symptoms of poisoning we focused on central nervous signs and symptoms, particularly on confusion, agitation, hallucinations, convulsions and consciousness level. The consciousness level was assessed by the Glasgow coma scale (GCS) and a GCS below 8 was considered as coma.

Data are presented as mean and range unless indicated otherwise. The Mann–Whitney test was used for categorical variables to determine whether any of differences of signs and symptoms among mushroom species were significant. A *p*-value of less than 0.05 was considered as significant. Analyses were carried out with the Statistical Package for Social Science 11.5 for Windows (SPSS).

3. Results

49 patients were enrolled in the study: 32 cases of *A. muscaria* poisoning and 17 cases of *A. pantherina* poisoning that were confirmed by mycological analysis. The mean age of the study cohort was 45 years (range, 19–77 years). There were 21 women and 28 men. In only one case *A. muscaria* was ingested intentionally due to its

hallucinogenic effects. In all other cases *A. muscaria* was mistaken for edible species and 75% of patients (25/32) claimed to have ingested *Amanita caesarea* (Caesar's mushroom). In all *A. pantherina* poisoning cases the mushrooms were mistaken for edible species. In 7 out of 17 cases (41%) patients claimed to have eaten *Amanita rubescens* (blusher), 3 patients mistook *A. pantherina* for *Macrolepiota procera* and one for *Agaricus*. All poisonings happened between July and October.

The first symptoms appeared 30 min to 2 h after mushroom ingestion. The symptoms and signs of 32 cases of *A. muscaria* poisoning and 17 cases of *A. pantherina* poisoning are presented in Table 1.

Patients poisoned with *A. muscaria* were significantly more often confused (26/32, *p* = 0.01) and agitated (20/32, *p* = 0.03) compared to those poisoned with *A. pantherina* (8/17 and 5/17, respectively). On the other hand, patients poisoned with *A. pantherina* were significantly more commonly comatose (5/17) compared to those poisoned with *A. muscaria* (2/32) (*p* = 0.03), but there was no difference in somnolence between the patients poisoned with *A. muscaria* (13/32) and *A. pantherina* (5/17) (*p* = 0.44). In *A. muscaria* poisoning somnolence frequently appeared after the onset of other symptoms like confusion, agitation, euphoria and hallucinations, while in *A. pantherina* somnolence and coma were presented on admittance. There was also no difference in hallucinations (10/32), myoclonus (6/32), convulsions (4/32), vomiting (18/32) and diarrhoea (5/32) in patients poisoned with *A. muscaria* compared to those poisoned with *A. pantherina* (7/17, 6/17, 2/17, 11/17 and 4/17, respectively) (*p* > 0.05) (Table 1). Cholinergic and anticholinergic symptoms were not observed in these patients who were treated with supportive care and gastric decontamination. Agitated patients were treated with benzodiazepines (10/31 in *A. muscaria* and 5/17 in *A. pantherina*, *p* = 0.89). All the patients survived and had no consequences of poisoning on discharge.

4. Discussion

This is the largest cohort of patients poisoned with *A. pantherina* and *A. muscaria* reported so far. In this study with 32 cases of *A. muscaria* poisoning and 17 cases of *A. pantherina* poisoning some significant differences in the clinical picture of patients poisoned with *A. pantherina* and *A. muscaria* were observed. In *A. muscaria* poisoning the

Table 1

Symptoms and signs after *A. muscaria* and *A. pantherina* ingestion in 49 patients treated in the Ljubljana Poison Control Centre in the past 33 years.

	<i>Amanita muscaria</i> (n = 32)	<i>Amanita pantherina</i> (n = 17)	<i>p</i> value
Confusion	26	8	0.01
Agitation	20	5	0.03
Hallucinations	10	7	0.49
Myoclonus	6	6	0.20
Convulsions	4	2	0.94
Somnolence	13	5	0.44
Coma	2	5	0.03
Vomiting	18	11	0.57
Diarrhoea	5	4	0.50

patients were more often agitated and confused, which may be related to the excitatory effect of ibotenic acid on the central nervous system and its higher concentration in comparison with muscimol that has a depressant effect. On the other hand, the higher frequency of coma in *A. pantherina* poisonings may be related to the depressant effect on the central nervous system of muscimol and its higher concentration in comparison with ibotenic acid (Magdalan and Antonczyk, 2007; Michelot and Melendez-Howell, 2003). In addition, many of the *A. muscaria* poisoned patients became somnolent in the later course of poisoning, which might be related to the fact that ibotenic acid spontaneously decarboxylates to muscimol in the brain (Nielsen et al., 1985; Tsujikawa et al., 2006). *A. muscaria* also contains muscazone which is a lactame isomer of muscimol that acts with a longer latency period (Satora et al., 2005). Nevertheless, somnolence did not differ between the two mushroom species.

Searching PubMed we have found only 8 cases of *A. muscaria* poisoned patients with a reported consciousness level of whom 3 were described as comatose (3/8), while 5 out of 8 published *A. pantherina* poisoned patients were comatose (5/8) (Brvar et al., 2006; Hohn and Schoenemann, 2000; Łukasik-Giebocka et al., 2011; Magdalan and Antonczyk, 2007; Satora et al., 2005, 2006). In the meta-analysis of patients included in this study (49) and the aforementioned published cases (16) coma became even more significantly common in *A. pantherina* (10/25) than in *A. muscaria* (5/40) poisoning ($p = 0.01$).

Accordingly, so-called ibotenic or pantherina-muscaria syndrome after *A. muscaria* and *A. pantherina* poisoning might be divided into two subtypes, which might be the result of the different contents and concentration ratios of ibotenic acid and muscimol in these two mushroom species since *A. muscaria* contains more excitatory ibotenic acid, while *A. pantherina* contains more inhibitory muscimol (Michelot and Melendez-Howell, 2003; Tsujikawa et al., 2006). In fresh *A. muscaria* the amount of ibotenic acid was estimated at 100 ppm and that of muscimol below 3 ppm (Lund, 1979). In dry caps of *A. muscaria* the amount of ibotenic acid was 10–2845 ppm and that of muscimol 46–1052 ppm, while in dry caps of *A. pantherina* the amount of ibotenic acid was 188–269 ppm and that of muscimol 1554–1880 ppm (Tsujikawa et al., 2006). However, the fleshy *Amanitas* might contain even more ibotenic acid since drying in the sun or with a heater increases the muscimol level by decarboxylation of ibotenic acid and much of the ibotenic acid is lost (Tsunoda et al., 1993). Furthermore, the amount of ibotenic acid and muscimol of the same *Amanita* species can differ regionally and periodically since in spring and summer mushrooms may contain up to 10 times as much ibotenic acid/muscimol compared to the autumn findings (Benjamin, 1992). Accordingly, the division of ibotenic syndrome remains hypothetical since the dose of toxic ingredients was unavailable in the majority of cases due to the unknown amount of ingested mushrooms and ibotenic acid/muscimol levels in the mushroom remains. Nevertheless, the proposed division of ibotenic syndrome is based on different toxic compound ratios in two mushroom species, not on their absolute amounts.

In this study all *Amanitas* ingestions happened in summer or autumn and three-quarters of patients mistook *A. muscaria* for edible *A. caesarea*, which is a mushroom protected by law in Slovenia, and almost half of the patients poisoned with *A. pantherina* picked up a mushroom presumed to be *A. rubescens*.

Accordingly in patients presenting neurological symptoms, especially after ingestion of mushrooms presumed to be *A. caesarea* or *A. rubescens* in summer or autumn, *A. muscaria* and *A. pantherina* poisoning should always be considered. Gastrointestinal and cholinergic/anticholinergic signs and symptoms are not characteristic and distinctive for the syndrome. The meta-analysis of these symptoms in *A. muscaria* and *A. pantherina* poisoning could not be done reliably due to the diverse descriptions of symptoms in the published cases.

The limitation of this study is also its retrospectiveness with a scattered description of symptoms, particularly regarding their time course. This could be overcome with the prospective approach and use of specific protocols for history and clinical examination of *A. pantherina* and *A. muscaria* poisoned patient on admission and during hospitalization.

In conclusion, *A. muscaria* containing more excitatory ibotenic acid results more often in confusion and agitation, while *A. pantherina* containing more inhibitory muscimol causes coma more often. Accordingly, the so-called ibotenic or pantherina-muscaria syndrome after *A. muscaria* and *A. pantherina* poisoning might be divided into two subtypes.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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Catching flies with *Amanita muscaria*: traditional recipes from Slovenia and their efficacy in the extraction of ibotenic acid

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Catching flies with *Amanita muscaria*: traditional recipes from Slovenia and their efficacy in the extraction of ibotenic acid

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Abstract

Ethnopharmacological relevance: Fly control is necessary for maintaining good hygiene on farms. Because organic farmers are skeptical about chemical pesticides, alternative fly control remedies are being considered. *Amanita muscaria* is a widespread fungus that contains ibotenic acid and muscimol. This fungus has been used to catch flies for centuries, but traditional recipes are poorly described, documented and characterized.

Aim of the study: The aim of the present study was to collect the traditional methods for preparing *A. muscaria* for catching flies in Karst and Gorjanci and to investigate the influence of different traditional methods on the release of ibotenic acid and muscimol from the fungal material.

Materials and methods: The research was conducted in villages in Karst and in the foothills of Gorjanci, Slovenia. Data regarding the traditional recipes of *A. muscaria* for catching flies were collected through structured interviews with 31 people in Karst and 28 in Gorjanci. Eight preparations were prepared based on traditional methods, and the amount of ibotenic acid and muscimol released from the fungal material at five different time points (0.5, 1, 2, 3 and 24 h) was determined by high-performance liquid chromatography (HPLC).

Results: Detailed descriptions of preparations used for catching flies were obtained from three informants in Karst, who were originally from other parts of Slovenia, and 13 informants in the foothills of Gorjanci. However, there were no reports regarding current usage. A total of 9 different methods were collected. Some methods were simple and included soaking in milk or water or dripping a little milk onto the mushroom. Others were more complex and included a combination of heat or mechanical processing and soaking in milk or water. For all preparations, the release of ibotenic acid was time-dependent, with the extracted amount increasing over time. Although milk was used more often than water in the traditional recipes, the release of both substances was not dependent on the solvent used. Fungal material that was exclusively soaked in water or milk released the smallest amount of ibotenic acid and muscimol at each time point. Additional heat and mechanical processing led to faster release of ibotenic acid and muscimol from the fungal material.

Conclusions: The tradition of using *A. muscaria* for catching flies was present in Gorjanci but not in Karst. The methods used to prepare the fungal material vary, and these differences are reflected in the release profile of ibotenic acid.

Abbreviations:

IBO, ibotenic acid; MUS, muscimol; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography

Keywords:

Amanita muscaria; Mushroom; Fungi; Fly control; Ibotenic acid; Muscimol

Chemical compounds studied in this article:

Ibotenic acid (PubChem CID: 1233); Muscimol (PubChem CID: 4266)

1 Introduction

Fungi are important sources of chemicals that have been used traditionally and have important potential for the future (Grienke et al., 2014; Lau et al., 2015). *Amanita muscaria* (fly agaric) has traditionally been used to catch flies. Flies irritate livestock and people, and they also transmit pathogens. To maintain good hygiene, farmers must control pathogens, including those transmitted by flies. Given that organic farmers are skeptical about the use of chemical control, other possibilities are being explored (Meerburg et al., 2007). *A. muscaria* is a widespread fungus in the northern hemisphere and grows in mycorrhizae with spruce and birch (Lang, 2015). As early as the 13th century, Albertus Magnus wrote that *A. muscaria* was used for killing flies (Crundwell, 1987). According to Scopoli (1772), the tradition of preparing *A. muscaria* for stupefying flies was utilized in the territory of Slovenia in the 18th century. Scopoli wrote: “On the top it is red and sprinkled with white outgrowths, other parts are completely white. It lives in forests of Carniola. It dies when eaten by worms, which have flattened its hat. This poisonous mushroom is used by housewives in Ljubljana, which eat it like Caesar's mushroom. All the flies, that try the mushroom, do not die but some are seized by drowsiness. After a few hours they wake up and live again. The juice of it can kill bed bugs.” Some Slovenian mushroom field guides (Kromar, 1995; Poler, 1986) and also copies translated from German (Gminder and Böhning, 2008; Lang, 2015) describe the use of *A. muscaria* for killing flies. The methods of preparation they mention include soaking in (sweetened) milk and cooking in milk. In the 19th century, Pouchet demonstrated (Pouchet, 1839 in Rubel and Arora, 2008) that the toxins in *A. muscaria* are water soluble and that boiling removes the toxins from the mushroom. He boiled caps of *A. muscaria* in water and gave the water extract and the boiled caps separately to dogs. Dogs that ate the boiled caps lived, whereas dogs that drank the water extract died.

Ibotenic acid (IBO) and muscimol (MUS; a decarboxylation product of IBO) are two water-soluble compounds from *A. muscaria*. These compounds were independently discovered in the 1960s by three research groups in Japan (Takemoto et al., 1964a, 1964b, 1964c), England (Bowden and Drysdale, 1965; Bowden et al., 1965) and Switzerland (Eugster et al., 1965; Mueller and Eugster, 1965). The Swiss researchers investigated the narcosis-potentiating action of these compounds, whereas the researchers from Japan and England focused on the flycidal or fly-stunning action of *A. muscaria*, respectively. It was later demonstrated (Catalformo and Eugster, 1970) that IBO and MUS have only weak insecticidal action towards the house fly, *Musca domestica*. *A. muscaria* also contains 1,3-diolein, a house fly attractant (Muto and Sugawara, 1970). IBO and MUS also affect the survival and performance of two frugivorous *Drosophila* flies, *Drosophila melanogaster* and *Drosophila immigrans* (Tuno et al., 2007). *A. muscaria* causes poisoning in humans most likely due to IBO and MUS. The symptoms are variable and include confusion, dizziness, visual and auditory aesthesia, space disorientation, unawareness of time, hallucinations and vivid color perception (Michelot and Melendez-Howell, 2003; Norton, 2001).

The traditional use of *A. muscaria* for catching flies has not been investigated in Slovenia and is only mentioned but not described in the available literature. Moreover, chemical analyses of

the different preparation methods have not been reported to date. This paper comprises a field study investigating the traditional use of *A. muscaria* in Slovenia and laboratory research investigating different traditional methods for preparing *A. muscaria* on the basis of the amount of IBO and MUS released from the fungal material. Such studies might lead to the discovery of new fly control remedies based on the knowledge of our ancestors (Verpoorte, 2015).

2 Methodology

2.1 Study area

Traditional use of *A. muscaria* was investigated in villages in the Karst plateau in southwestern Slovenia and in villages in the foothills of the Gorjanci mountain range in southeastern Slovenia (Figure 1). The villages in the Karst plateau included Pliskovica, Veliki dol, Brje pri Komnu, Gorjansko, Klanec pri Komnu, Štanjel, Kobdilj, Grahovo brdo, Griže and Štjak. The villages in the foothills of Gorjanci included Igljenik, Vrhe, Dolž, Mali Cerovec, Pangrč Grm, Sela pri Zajčjem Vrhu, Stopiče, Dolenji Suhadol, Cerov Log and Mihovo.



Figure 1: Map of the study area. The villages where the interviews were conducted are represented by dots.

The Karst region is an extensive limestone plateau in southwestern Slovenia that continues beyond the national border on the Italian side. This transition region has Mediterranean and continental influences (Brancelj, 1999). The mean annual precipitation ranges from 1400 to 1650 mm (Petrič, 1999), and the mean January and July temperatures are 2.4 °C and 20.8 °C, respectively. The predominant bedrock is limestone, which is vulnerable to corrosion. The bedrock is covered with chromic cambisol, chromic luvisol and rendzina. Pubescent oak (*Quercus pubescens*), hophornbeam (*Ostrya carpinifolia*) and flowering ash (*Fraxinus ornus*) (Zupančič, 1999) have major roles in the natural forests. In the 19th century, parts of Karst were afforested with black pine (*Pinus nigra*). In 1991, the population density was 41 people per km², and the farming population represented 4.1 % of the total population. Altogether, there are 100 settlements (Brancelj, 1999). According to fungi mapping data in Slovenia,

Amanita muscaria var. *muscaria* (L.) Hook. (1797) (Boh et al., 2015) and *Amanita muscaria* var. *aureola* Kalchbr. (1873) (Poler et al., 2015) are only present in the southeastern part of the Karst plateau near Sežana.

The Gorjanci region is a 45-km long mountain range in southeastern Slovenia that runs from southwest to northeast along the border with Croatia. This region has a moderate continental climate with mean annual precipitation between 1100 to 1300 mm. In the foothills, the mean January and July temperatures are approximately 0 °C and 20 °C, respectively. The Gorjanci Mountains primarily consist of limestone and dolomite and covered with rendzina and chromic cambisol. The higher regions of Gorjanci are overgrown with extensive beech forests (*Fagus sylvatica*). Some areas were deforested for pasture, but they are now becoming naturally overgrown or reforested with spruce (*Picea abies*) and fir (*Abies alba*). Forests of beech (*Fagus sylvatica*) and hophornbeam (*Ostrya carpinifolia*) grow in the eastern part of Gorjanci. Oak forest (*Quercus petraea*) grows in the western and middle parts of Gorjanci up to 400 and 600 m altitude, respectively. The land area is sparsely populated given the unfavorable terrain; the population density is 27 people per km² (1991). There are 87 settlements in total, most of which are located on the periphery. In 1991, the farming population represented 22.2 % of the total population (Perko, 1999). According to fungi mapping data in Slovenia, *Amanita muscaria* var. *muscaria* (L.) Hook. (1797) is widespread in the Gorjanci region (Boh, 2015), but *Amanita muscaria* var. *aureola* Kalchbr. (1873) is not present (Poler, 2015).

2.2 Field interviews

The use of *A. muscaria* was investigated in the Karst and Gorjanci regions through interviews with local people. The interviews were conducted during the periods of October to December 2013 and May to September 2014. In the study, 31 and 28 people were interviewed in Karst and Gorjanci, respectively. Their age ranged from 33 to 89 years with an average age of 61 years in Karst and 68 years in Gorjanci (See Table 1). In Karst, 23 informants were originally from the Karst region, 3 were born near that region and 5 were from other parts of Slovenia. In Gorjanci, 20 informants were originally from villages in the foothills of Gorjanci, and 8 informants were born close to that region (radius 30 km). No informant was from other parts of Slovenia. Informants were contacted in villages (on the street, outside their house or in the field). The informants often recommended that we interview people more knowledgeable in their family, village or neighboring village. The informants were recruited without any selection based on their gender, age or social status. The interviews occurred in the houses of informants and in the fields. The language used in the interviews was Slovenian. The informants were made aware of the scope of the study, and prior informed consent was obtained.

The informants were requested to answer the following questions:

- “Do you know of *A. muscaria*” (Original Slovene question: “Ali poznate rdečo mušnico?”)

- “Have you ever heard of the use of *A. muscaria*?” (Original Slovene question: “Ali ste že kdaj slišali za uporabo rdeče mušnice?”)
- “How is *A. muscaria* prepared?” (Original Slovene question: “Kako ste pripravljali rdečo mušnico?”)

Table 1: Informants' age and gender in Karst and Gorjanci.

		Karst	Gorjanci
Average age (years)		61	68
Age (years)	33-49	7	4
	50-69	16	7
	70-89	9	17
Gender	male	9	4
	female	22	24

2.3 Chromatographic determination of ibotenic acid and muscimol in preparations of *A. muscaria*

2.3.1 Reagents and chemicals

Water (JT Baker, Deventer, Netherlands) and acetonitrile (JT Baker, Deventer, Netherlands) used for high-performance liquid chromatography (HPLC) analysis were of HPLC-grade purity. Heptafluorobutyric acid (Sigma-Aldrich, Steinheim, Germany) with ion chromatography-grade purity was used as an ion-pairing reagent for HPLC analysis. Methanol (Sigma-Aldrich, Steinheim, Germany) used for extraction was p.a. (pro analysi) purity. IBO (purity 98 %) and MUS (purity 99 %) were purchased from Abcam (Cambridge, United Kingdom). Milk with 3.5 % milk fat (Ljubljanske mlekarne, Ljubljana, Slovenia) and demineralized water were used for the preparations. Fermentation-produced SHY-MAX Plus chymosin (200 IMCU/ml) was used for milk clotting.

2.3.2 Standard solutions and calibration curves

Stock standard solutions were prepared by dissolving the appropriate amount of pure IBO and MUS in water to yield a final concentration of 1000 µg/ml. Stock standard solutions were stored at -20 °C. Stock standard solutions were further diluted with water to obtain a series of standard solutions with concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.1, and 0.05 µg/ml for IBO as well as 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.1, and 0.05 µg/ml for MUS. Calibration curves were constructed by plotting the peak areas of the compounds (y) against their concentration (x). Regression analysis of the data for each component provided the slope, intercept and correlation coefficient for each calibration curve. The calibration curves were used to quantify IBO and MUS in the samples.

2.3.3 Fungal material

The analyses were performed on fly agaric mushrooms (*Amanita muscaria*; common local and also Slovene name: rdeča mušnica) that had been harvested at Črni vrh (45°55'19.42"N, 14°2'43.04"E), Slovenia in October 2014. All *A. muscaria* mushrooms were macroscopically identified. To prepare the *A. muscaria* samples, three caps (weighing 48 to 126 g) were sliced into ten equal pieces. Eight pieces from each cap were immediately prepared following traditional and modified methods (one piece for each of the 8 methods), and two pieces were stored at -20 °C for analysis of the IBO and MUS contents. For method validation, *A. muscaria* caps were weighed and stored at -20 °C. A voucher specimen (F100) was dried and deposited at the herbarium of the Faculty of Pharmacy, University of Ljubljana.

2.3.4 *A. muscaria* preparations

Seven preparations (P1-P7) were made according to the results of field interviews (See Table 2). Preparations P3 and P4 were heated to boiling on a hotplate. Preparation P8 was similar to P7 with one modification; instead of warm milk (40 °C), room temperature milk was added to the sliced fungal material. Pieces of similar sizes (see Chapter 2.3.3) were used, ranging in mass from 5.7 to 10.0 g for the first cap, from 3.3 to 5.6 for the second cap and from 10.8 to 14.2 for the third cap of *A. muscaria*. In all preparations, the fungal material was placed in a 400-ml beaker and prepared according to a traditional or modified method using 50 ml of milk or water. The beaker was covered with aluminum foil to prevent solvent loss. The preparations were sampled at five different time points: 0.5, 1, 2, 3 and 24 h. The beaker was mixed gently, and 1 ml of the medium was transferred into a 2-ml microcentrifuge tube. Samples were stored at -20 °C. After thawing, milk clotting was induced by adding 460 µl of chymosin solution (26 IMCU/ml) to 1 ml of sample in a microcentrifuge tube and placing it into a water bath at 34 °C for 30 min. The samples were centrifuged at 15000 rpm for 15 min. The supernatant was filtered using a 0.22-µm Nylon Syringe Filter Q-Max RR 25 mm (Frisenette, Knebel, Denmark) prior to HPLC analysis. Aqueous samples were thawed and filtered using a 0.22-µm Nylon Syringe Filter Q-Max RR 25 mm (Frisenette, Knebel, Denmark) prior to HPLC analysis.

2.3.5 Extraction of ibotenic acid and muscimol from *A. muscaria*

Two pieces of each mushroom cap were used to determine the concentrations of IBO and MUS, and the other eight pieces of the same cap were assumed to have the same concentration of IBO and MUS. Two pieces of each mushroom cap were freeze-dried (Freeze dryer Lio – 2000, Kambič, Slovenia), weighed and then dry-milled with an IKA A10 laboratory mill (IKA Laboratory Technology, Staufen, Germany). Powdered fungal material (50 mg) was macerated in 10 ml of 50 % methanol for 2 h on a shaker at 120 rpm at room temperature and later centrifuged for 15 min at 5000 rpm. A 5 ml aliquot of the supernatant was dried on a rotary evaporator at 40 °C and then resuspended in 2 ml of HPLC-grade purity

water. Prior to HPLC analysis, the sample was filtered using a 0.22- μ m Nylon Syringe Filter Q-Max RR 25 mm (Frisenette, Knebel, Denmark).

2.3.6 Method validation

The extraction of IBO and MUS from *A. muscaria* was repeated several times with different *A. muscaria* caps to assess repeatability. Three frozen *A. muscaria* caps were freeze-dried (Freeze dryer Lio – 2000, Kambič, Slovenia) and then separately dry-milled with an IKA A10 laboratory mill (IKA Laboratory Technology, Staufen, Germany), and three samples (50 mg) were prepared from the powdered material of each mushroom. Samples were extracted with the method described in 2.3.3. Repeatability was expressed as the coefficient of variation (CV, %) of the experimental values for each mushroom specimen.

To assess the amount of IBO and MUS that remained in the fungal material after extraction, three samples (50 mg) of freeze-dried and powdered *A. muscaria* were extracted twice with 10 ml of 50 % methanol. After the first extraction and centrifugation, as much of the extract was removed as possible by decantation, and 10 ml of fresh 50 % methanol was added. The second extraction also followed the method described in 2.3.5. The re-extraction efficiency was expressed as the ratio (%) of the extracted IBO in the second extraction to the sum of the extracted IBO in the first and second extractions.

The biological variability of different pieces of the same cap of *A. muscaria* was assessed by extraction of IBO and MUS from three pieces of the same cap. Three frozen pieces of *A. muscaria* caps were freeze-dried and cut into 3 equal pieces each, and each of the obtained 9 pieces was dry-milled separately. Samples (50 mg) of powdered fungal material were extracted with the method described in 2.3.3. Biological variability was expressed as the coefficient of variation (CV, %) of the experimental values for each mushroom specimen.

In recovery experiments, known amounts of IBO and MUS (300 μ g) were spiked into three samples containing 50 mg of powdered fungal material that was suspended in 10 ml of 50 % methanol. The sample preparation procedure was also evaluated in an additional experiment in which known amounts of IBO and MUS (300 μ g) were spiked into three blanks containing only 10 ml of 50 % methanol. The concentrations of IBO and MUS in both experiments were determined following the method described in 2.3.5.

2.3.7 Instrumentation and chromatographic conditions

The HPLC system (Shimadzu Prominence) used for analysis consisted of a system controller (CBM-20A), a solvent delivery pump with a degasser (DGPU-20A5), an autosampler (SIL-20AC), a column oven (SPO-20AC) and a photodiode array detector (SPD-M20A). The data were recorded using LabSolution Software. Separation was performed using a Kinetex C18 (250 $\times$ 4.6 mm i.d., 5 μ m particle size), and the column temperature was maintained at 15 $^{\circ}$ C throughout the separation. UV absorbance was measured at 220 nm with a 1.2-nm slit width.

For the separation, we used a mobile phase containing 8 mM HFBA in water, which was previously described (Merova et al., 2008). The chromatographic conditions were as follows: 100 % 8 mM HFBA in water, 0.8 ml/min flow for 20 min. After each analysis, the column was flushed with 50 % acetonitrile in water for 15 min at 1.5 ml/min. The column was reconditioned with the initial eluent for 20 min. The presence of IBO and MUS in the samples was confirmed by the retention times of the standards at 3.6 and 10.5 min accordingly.

2.3.8 Calculations

Two pieces of each *A. muscaria* cap were used to determine the concentrations of IBO and MUS in the fresh fungal material, which were calculated from their concentration in the freeze-dried fungal material and the water loss during freeze-drying. The concentrations of IBO and MUS were expressed as the average of the experimental values in two pieces of *A. muscaria*. All pieces from the same cap were assumed to have the same concentrations of IBO and MUS.

The amounts of IBO and MUS in each piece of *A. muscaria* cap used as starting material for the preparations were calculated from the concentrations in the fresh fungal material and the mass of each piece. The release of IBO and MUS from the fungal material into milk or water was monitored over 24 h, and the preparations were sampled at five different time points: 0.5, 1, 2, 3 and 24 h. The liquid preparations contained IBO and MUS that originated from *A. muscaria*, but MUS could also be formed from IBO during extraction. For each time point, the proportion of IBO released was calculated as the amount of IBO released up to that time point divided by the sum of the calculated content of IBO and MUS in a piece of fresh *A. muscaria*. Similarly, for each time point, the proportion of MUS released was calculated as a quotient of the amount of MUS released up to that time point divided by the sum of the calculated content of IBO and MUS in a piece of fresh *A. muscaria*. The loss of solvent due to sampling (1 ml sample aliquot at each time point from a 50 ml extraction volume) was included in the calculations. The proportion of IBO and MUS released from the fungal material into milk or water was expressed as the average of the experimental values from three individual experiments.

3 Results and Discussion

3.1 Traditional use of *A. muscaria* in Karst and Gorjanci for catching flies

In the present study, traditional use of *A. muscaria* was investigated in villages in Karst and the foothills of Gorjanci. In Karst, 29 out of 31 informants confirmed that they were aware of *A. muscaria*, but only 3 reported the use of *A. muscaria* for catching flies and provided a detailed description of the preparations used (Table 3). These three informants were originally from other parts of Slovenia and remembered the use from their home. One informant was from the small village of Igljenik located in the foothills of Gorjanci. Three other informants in Karst also reported recreational use of *A. muscaria* as a drug, which they remembered from

their student life in Ljubljana. In Gorjanci, all 28 informants were aware of *A. muscaria*, 17 reported its use for catching flies and 13 provided a detailed description of the preparations (Table 3). Among these 13 informants, 12 were originally from villages in the foothills of Gorjanci, and one moved from the town of Novo mesto to one of these villages and observed the preparation at a neighbor's house. However, no reports about the present use of such preparations were obtained. These results suggest that the tradition of preparing *A. muscaria* for catching flies was present in the foothills of Gorjanci but not in Karst, despite the fact that almost all the informants were aware of *A. muscaria*. This disparity might be attributed to the scarcity of *A. muscaria* in Karst and its prevalence in Gorjanci. Traditional knowledge, which is still present, will likely disappear in the coming years given the lack of reports about current use. Modern methods for fly control are accessible and have already replaced traditional methods. As one of the informants noted, "Nowadays, sprays and fly tapes are available, and we do not do that anymore".

The informants reported two other possibilities for fly control. One informant in Gorjanci reported the use of the mushroom *Phallus impudicus*. The entire unprocessed mushroom is used to attract the flies, which stay near the mushroom and do not enter the barn. One respondent from Karst and one from Gorjanci reported the use of glue made from cooked mistletoe fruit. Both informants used a Slovenian name, "bela omela", which most likely referred to *Viscum album*, or less likely to *Viscum abietis* or *Loranthus europaeus*. The glue is applied on the surface of a stick, and the flies are caught on the adhesive surface. The use of ferns (most likely from the genus *Dryopteris* or *Pteridium*) was also mentioned. Sweetened milk is used to wet the fern, which is subsequently hung out for the flies. Flies are caught on the fern, which is subsequently placed in a bag. Trampling on the bag kills the flies.

In Karst, three older women were the only informants to report a tradition of preparing *A. muscaria* for catching flies. Each informant reported a different method. This difference may be because the informants were born in different parts of Slovenia and brought this particular knowledge from their homes. Interestingly, in the foothills of Gorjanci, six different methods were reported by 13 informants, all of whom were born and live in this limited area. Some methods are simple and involve only soaking in milk or water or dripping a little bit of milk onto a cap. In other methods, *A. muscaria* is prepared with a combination of heat or mechanical processing and soaking (Table 2). There was one report that described preparing *A. muscaria* exclusively with heat processing and without any solvent (Table 2: Informant 12). On the other hand, the method included the use of milk in 13 descriptions and water in two descriptions (Table 2). The informants did not report sugar as an ingredient, which is likely a result of poverty. "Sugar and milk attracted the flies, but the poor had only milk. It is also better to use sugar if you own a cat" (Personal communication with Prof. Dr. Franc Pohleven from Biotechnical Faculty, University of Ljubljana, 2014).

Table 2: Grouped methods of preparing *A. muscaria* for catching flies reported in Karst (respondents' descriptions 1 to 3) and Gorjanci (respondents' descriptions 4 to 16). The last column presents abbreviations of the preparation (P) with which the corresponding methods were tested.

Groups of methods	Traditional methods	Informants' descriptions	Preparation tested
Simple methods (only solvent used)	Soak in milk	4, 5, 6, 7, 8	P1
	Soak in water	9	P2
	A little bit of milk is dripped on the cap	10, 11	Not tested
Heat processing method	Fungal material is placed on a stove to soften	12	Not tested
Heat processing methods and soaking	Boil in milk	2, 13	P3
	Boil in water	14	P4
	Fungal material is placed on a stove to soften and milk is poured on it	1	P5
Mechanical processing methods and soaking	Fungal material is tapped on lamellae side, and milk poured on it	15, 16	P6
	Fungal material is sliced in pieces, and warm milk is poured on it	3	P7

Table 3: Informants' descriptions of different methods for preparing *A. muscaria* to catch flies

Informants' descriptions			Methods
Karst	1	When I still lived at home we gathered fly agaric mushrooms. We placed a fly agaric on a warm stove to soften it, and then we poured milk on it. We had it in a small bowl. This was poison for the flies. The flies were attracted to it, drank the milk and died. We did this back at home but here people do not do this. (Woman, born 1939 in Igljenik, interviewed in Veliko Polje)	Heat processing and soaking
	2	Back home we soaked a fly agaric mushroom in milk and boiled it on a stove to release water. The milk took on a beautiful scent. We placed it where there were many flies and away from children. (Woman, born 1931 in Žetale pri Ptuj, interviewed in Sveto pri Komnu)	
	3	My mother thinly sliced fly agaric mushrooms, chopped them like onions and put them into a small container. She poured warm milk on them and placed the container where there were many flies. The flies drank the milk and were poisoned. (Woman, born 1944 in Žalna pri Grosuplju, interviewed in Štjak)	Mechanical processing and soaking
Gorjanci	4	Fly agaric was used for catching flies. The cap of the mushroom was placed into a container and a little bit of milk was poured on it, so the cap could swim. Most likely the poison was released into the milk. When the flies came and drank the milk, they dropped dead. One could soak the whole cap or cut in pieces. The upper red part of the cap had to face up because the red color attracted the flies. It soon began to stink and was therefore useful only for a day or two. You had to put it in a place where a cat could not reach it. Nowadays, sprays and fly tapes are available, and we do not do that anymore (Man, born 1960 in Vrhe pri Dolžu, interviewed in Dolž).	Simple methods
	5	Fly agaric was placed on plate and little bit of milk was poured on it, approximately half a deciliter. This was set for the flies. (Woman, born 1929 in Veliki Cerovec, interviewed in Dolž).	
	6	Fly agaric was used for catching flies. The whole cap of a fly agaric was placed on a plate and milk was poured on it. The plate was kept on a table. Flies came, drank the milk and died. We did not use the fly agaric but I think the neighbors used it a lot. (Woman, born 1962 in Novo mesto, interviewed in Mali Cerovec)	
	7	I heard that milk was poured on the cap of a fly agaric mushroom and used for catching flies. (Woman, born 1942 in Gabrje, interviewed in Mihovo)	
	8	I remember that a cap of the fly agaric mushroom was placed in a shallow container with a little bit of milk in it. It was used for catching flies. The milk attracted the flies, and they were poisoned because of the fly agaric mushroom. We did not do this at home. (Woman, born 1927 in Dolenji Suhadol, interviewed in Dolenji Suhadol)	
	9	We set a fly agaric mushroom for catching flies but when our cat died, we did not do that anymore. We placed a cap of a fly agaric mushroom into a dish and poured water on it. We did not use milk because it breaks down the poison. My mother prepared the fly agaric mushroom. (Woman, born 1935 in Pangrč Grm, interviewed in Pangrč Grm)	
	10	Fly agaric was used for catching flies. My grandmother dripped a little bit of milk on the cap of a fly agaric mushroom. (Woman, born 1955 in Dolž, interviewed in Dolž)	
	11	My mother put the cap of a fly agaric mushroom on a plate and dripped milk onto it. When the	

		milk turned sour, we threw it away. (Woman, born 1936 in Stopiče, interviewed in Stopiče)	
12		We placed a cap of a fly agaric on paper, put it on a stove to soften a little bit and then on a shelf for the flies. The flies came, licked the fly agaric and they all died there. When there were many dead flies, you had to throw them away and put the fly agaric mushroom back on the shelf. Everybody in the village did this (i.e., set the fly agaric mushrooms for flies) (Woman, born 1929 in Veliki Cerovec, interviewed in Vrhe pri Dolžu)	Heat processing
13		Fly agaric mushroom was cooked in milk to release the poison and then it was set for the flies. You had to put it in a place where the cats could not reach it. (Woman, born 1933 in Mihovo, interviewed in Mihovo)	Heat processing and soaking
14		I heard that the fly agaric mushroom was cooked in water, and the water was then set for the flies. (Woman, born 1946 in Pangrč Grm, interviewed in Pangrč Grm)	
15		As children, we gathered fly agaric mushrooms when we grazed cattle. Our mother placed it on a plate or into a bowl and tapped it so the milk could get inside. She used a little bit of milk, maybe half a glass. There was no effect because there were too many flies. (Woman, born 1935 in Koroška vas, Mali Cerovec)	Mechanical processing and soaking
16		My father told me that you have to tap the cap of a fly agaric mushroom on the lower side and pour milk on it. This was then set for catching flies. (Man, born 1937 in Zajčji Vrh pri Stopičah, interviewed in Zajčji Vrh pri Stopičah)	

3.2 Determination of ibotenic acid and muscimol in *A. muscaria* preparations

3.2.1 Calibration curve

Two calibration curves were used to determine the concentration of IBO in the samples. A linear relationship of peak area over the concentration range of 0.05 to 12.5 µg/ml ($y = 18,339x + 165$; $R^2 = 0.99995$) was used for samples with low IBO content, whereas a linear relationship of peak area over the concentration range of 12.5 to 200 µg/ml ($y = 16,943x + 40,902$; $R^2 = 0.9997$) was used for samples with high IBO content. The response of muscimol over the concentration range of 0.05 to 100 µg/ml was linear ($y = 16,271x + 92$) with a correlation coefficient (R^2) of 0.9997. Limit of detection (LOD) and limit of quantification (LOQ) values were estimated from the signal to noise ratio. These values were 0.03 and 0.12 µg/ml for IBO and 0.06 and 0.19 µg/ml for MUS, respectively. Some concentrations of IBO and MUS were less than the LOQ and were considered as zero.

3.2.2 Method validation

Table 4 indicates the repeatability of the method (extraction and chromatography) for the determination of the IBO and MUS concentrations in *A. muscaria*. The CV was between 0.8 and 1.2 % for IBO and 2.1 and 7.2 % for MUS for three different caps. Table 4 and Table 5 show that six individual caps differ in the contents of both compounds. The CV for the between-cap biological variation was 36 % for IBO and 23 % for MUS.

Each line in Table 5 indicates the within-cap biological variation (biological variation in different pieces of the same cap) of IBO and MUS concentrations in the three different caps of *A. muscaria*. The CV for the within-cap biological variation was between 4.4 and 11.6 % for IBO and 7.2 and 18.3 % for MUS.

The re-extraction yield was $4.5 \% \pm 0.2 \%$ for IBO. This is partly due to our inability to completely remove the first extract from the fungal sample before adding the fresh solvent for re-extraction. The efficiency of the first extraction was therefore greater than 95%. The re-

extraction yield for MUS could not be assessed because the concentration of MUS in the second extraction was less than the LOQ.

The sample preparation procedure was evaluated by analyzing the recovery of three samples (50 mg of powdered fungal material) and three blanks spiked with the known amounts of IBO and MUS (300 µg). The mean recoveries were 98.5 ± 4.9 % for IBO and 100.3 ± 4.6 % for MUS in the samples and 99.3 ± 3.8 % for IBO and 99.2 ± 5.3 % for MUS in the blanks.

Table 4: Repeatability evaluated using three *Amanita muscaria* caps. The proportions of dry weight were 7.1, 6.5 and 6.2 % for caps 1, 2 and 3, respectively. IBO and MUS denote ibotenic acid and muscimol, respectively.

Compound	Cap No.	Concentration (mg/g dry weight)	Concentration (mg/g fresh weight)	CV (%)
IBO	1	6.62 ± 0.08	0.477 ± 0.006	1.2
	2	5.01 ± 0.04	0.327 ± 0.003	0.8
	3	5.59 ± 0.06	0.351 ± 0.004	1.1
MUS	1	0.367 ± 0.008	0.0261 ± 0.0006	2.2
	2	0.292 ± 0.012	0.0191 ± 0.0007	4.0
	3	0.456 ± 0.032	0.0286 ± 0.0021	7.2

Table 5: Within-cap biological variation evaluated using different pieces of three *Amanita muscaria* caps. The proportion of dry weight was 9.4, 11.5 and 11.4 % for caps 4, 5 and 6, respectively. IBO and MUS denote ibotenic acid and muscimol, respectively.

Compound	Cap No.	Concentration (mg/g dry weight)	Concentration (mg/g fresh weight)	CV (%)
IBO	4	8.16 ± 0.11	0.770 ± 0.034	4.4
	5	12.31 ± 0.76	1.414 ± 0.087	6.1
	6	6.55 ± 0.76	0.749 ± 0.087	11.6
MUS	4	0.328 ± 0.014	0.031 ± 0.004	13.8
	5	0.305 ± 0.022	0.035 ± 0.003	7.2
	6	0.228 ± 0.042	0.026 ± 0.005	18.3

3.2.3 Concentration of ibotenic acid and muscimol in fresh *A. muscaria* used for preparations

Three fresh *A. muscaria* caps were used for preparations based on traditional methods. The concentrations of IBO and MUS in the dried caps from Slovenia ranged from 6.38 to 11.21 mg/g and 0.23 to 0.69 mg/g, respectively. Fresh caps contained between 5.3 and 6.5 % of dry weight; therefore, the concentration of both compounds was roughly 15 times smaller in fresh caps and ranged from 0.4 to 0.7 mg/g (0.04 - 0.07 %) for IBO and 0.015 to 0.037 mg/g (0.0015 - 0.0037 %) for MUS. The concentrations of IBO and MUS in fresh *A. muscaria* correspond to a previous report (Tsunoda et al., 1993). Some other researchers also reported similar concentrations of IBO; however, the concentrations of MUS were up to 30 times higher (Gennaro et al., 1997; Tsujikawa et al., 2007). This might be due to degradation of IBO into MUS during harvesting, drying or extraction. Similarly, in *A. muscaria* preparations MUS originated from the fungal material and could also have been formed from IBO during extraction and before analysis. Therefore, IBO and MUS were treated as a single entity. The sum of IBO and MUS concentrations in the fresh *A. muscaria* caps that ranged from 2.62 to 4.47 µmol/g (see the last column of Table 6) was used as the initial concentration from which

IBO and MUS were released from the fungal material into the medium in the *A. muscaria* preparations.

Table 6: Mass, dry weight percentage and ibotenic acid and muscimol concentrations of three *A. muscaria* caps. The concentration of ibotenic acid and muscimol in the fresh caps was calculated from the concentrations that were measured in dried pieces of *A. muscaria* caps. IBO and MUS denote ibotenic acid and muscimol, respectively.

Cap No.	Mass (g fresh weight)	Dry weight percentage (%)	IBO (mg/g fresh weight)	MUS (mg/g fresh weight)	IBO and MUS (μ mol/g fresh weight)
7	73	6.3	0.706	0.035	4.47
8	48	5.3	0.439	0.037	2.78
9	126	6.5	0.414	0.015	2.62

3.2.4 Release of ibotenic acid and muscimol from *A. muscaria* in preparations based on traditional methods

In all preparations, the release of IBO from the fungal material was time-dependent, with the amount of released IBO gradually increasing over time. The preparations also contained MUS, which originated from the fungal material or was formed from IBO on site. The amount of MUS also increased over time, except in the preparations where the fungal material was boiled in milk or water. In boiled preparations, the heat potentially inactivates the enzymes and prevents the conversion of IBO to MUS. Comparison of the results from preparations made by soaking or boiling the fungal material in milk or water (Figure 2, preparations P1-P4) suggests that the release of IBO and MUS probably did not depend on the solvent used in the experiment given that no significant differences were observed between the results obtained from preparations P1 and P2 as well as between P3 and P4. A likely explanation is that IBO and MUS are water-soluble moieties and milk contains ca. 90 % water. These results also suggest that other components from milk are likely not involved in the release of IBO and MUS from the fungal material. Milk was reported more often than water (see Table 2), suggesting that milk acts as an additional attractant for the flies.

The method by which the fungal material was prepared strongly influenced IBO release. Preparations P1 and P2, which were generated by soaking the intact fungal material, exhibited the lowest proportion of released IBO at each time point. On the other hand, heat and mechanical processing of the fungal material led to faster extraction of IBO into milk or water (see Figure 2). In the first 30 min, the soaked intact fungal material released up to 10 % of its IBO content, the mechanically processed material released 10 to 25 % and the heat-processed material released at least 60 %. Heat processing also weakly influenced the yield of extraction. After 24 h, the heat processed fungal material released at least 85 % of its IBO content. When prepared with other methods, the proportion did not exceed 80 %. These results suggest that the fungal material was further disintegrated after additional processing and that increased amounts of IBO could be released into the solvent. Heat processing, especially boiling, likely led to more extensive disintegration than the other methods. The

results are also consistent with Pouchet's experiment (Pouchet, 1839 in Rubel and Arora, 2008), which proved that boiling could completely remove toxins from *A. muscaria*.

The preparation method also influenced the release of MUS into the solvent (see Figure 2). In the first 30 min, the soaked fungal material released up to 0.5 % of its MUS content, the heat-processed material released approximately 3 % and the mechanically processed material released from 0.5 to 2 %. Interestingly, the final yield of MUS ranged from 8.5 to 10 % in all preparations except where the fungal material was boiled in water or milk, where the yield was approximately 4 %. Although major differences in the release of IBO were noted between the heat processing and the mechanical processing methods, minor differences were also observed in each individual group. In the heat-processing group, in the first 3 h at each time point softening the fungal material on a hot plate (P5) had a slightly weaker effect on the release of IBO compared with boiling (P3 and 4) in the first 3 h at each time point. After 24 h, the proportion of released IBO was similar between the groups. In the mechanical processing group, the fungal material that was pressed on the lamellae side (P6) released slightly more IBO at each time point than the fungal material that was sliced into pieces (P7 and P8). No significant differences in IBO release were noted between the traditional method (P7) where the fungal material was sliced into pieces and warm milk was added and the modified method (P8) in which room temperature milk (25 °C) was added.

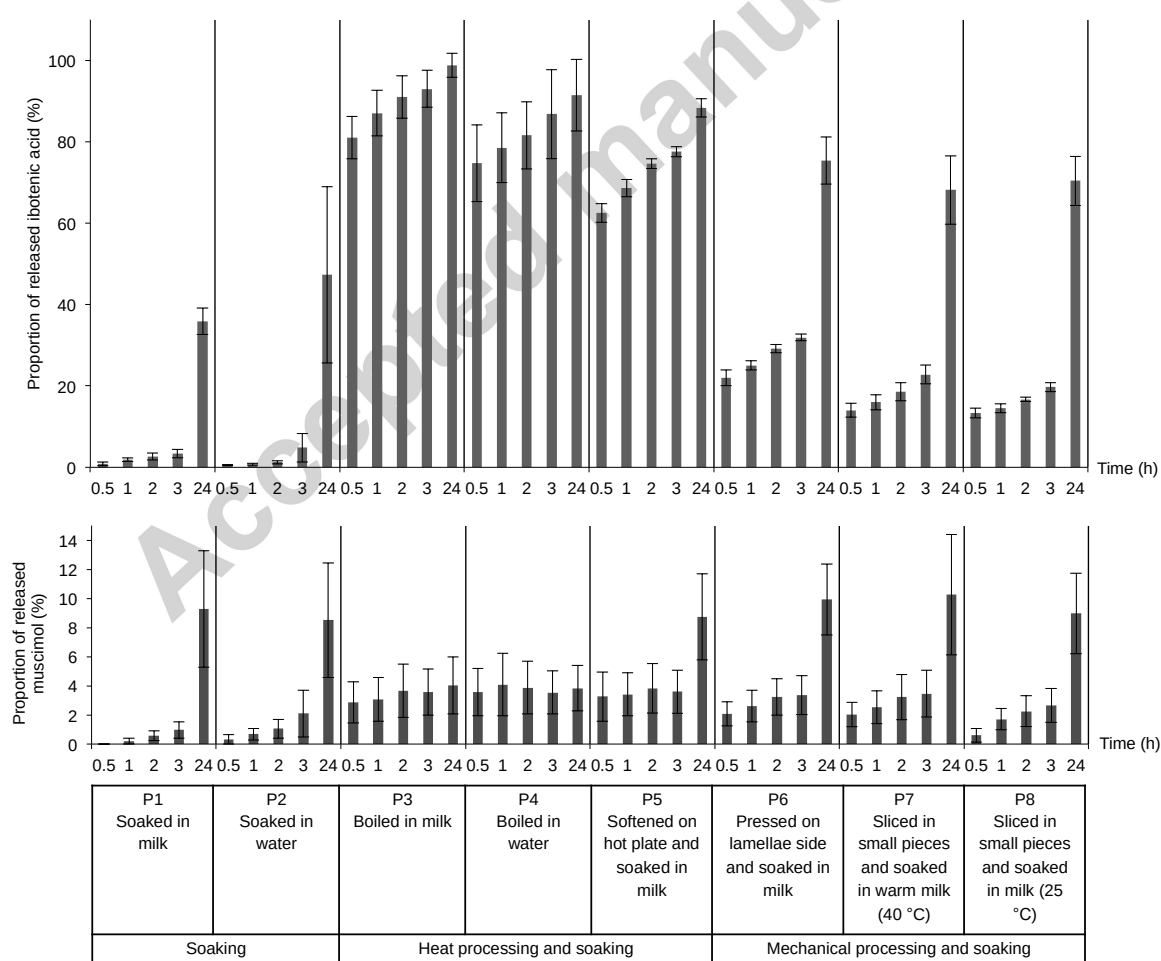


Figure 2: Release of ibotenic acid and muscimol from *Amanita muscaria* in preparations based on traditional methods. The preparations were sampled after 0.5, 1, 2, 3, and 24 h. The columns represent the proportion of released ibotenic acid or muscimol from the fungal material up to the time of sampling. The proportion of released IBO or MUS was calculated as a quotient of the amount released up to the time of sampling and the sum of the initial ibotenic acid and muscimol content in a piece of fresh *A. muscaria*. The released proportions of ibotenic acid and muscimol were calculated from three individual experiments. Error bars represent the standard error of the mean. P1 to 8 denote the eight different preparations.

3.3 Conclusions

Results from field interviews conducted in Karst and Gorjanci indicate that the traditional use of *A. muscaria* for catching flies was present in the foothills of Gorjanci but not in Karst. Traditional knowledge still exists, but it will likely be lost in the next years given that reports regarding current usage were not provided. Informants reported 9 different methods for preparing *A. muscaria* for catching flies. Some methods were simple, and only milk or water was needed to prepare the fungal material. Some methods were more complex and included additional heat or mechanical processing. Eight preparations were performed according to the traditional methods, and the release of IBO and MUS from the fungal material was determined by HPLC. The results indicate that the release of IBO and MUS did not depend on the solvent (milk or water) used in the experiment and that thermal and mechanical processing led to faster extraction of IBO and MUS.

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Graphical abstract

