THE PIGMENTS OF *HYGROPHORUS* SECTION *HYGROCYBE*, AND THEIR SIGNIFICANCE IN TAXONOMY AND PHYLOGENY

A Dissertation Presented

By

William Ganley Cibula

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BOTANY

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Approved as to style and content by:

Howard E. Bigelow

Dr. Howard E. Bigelow, Chairman of Committee

Dr. Arthur I. Stern, Member

Dr. Earl McWhorter, Member

Earl McWhorter

Edward L. Davis

Dr. Edward L. Davis, Department Head

Botany Department

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and Mrs. Marjorie Smith typed the final draft. Mrs. Karen Nelson typed the final copy and helped with many administrative matters as I was not on campus.

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Picayune, Mississippi

August, 1976

ABSTRACT

The Pigments of Hygrophorus section Hygrocybe
and their Significance in Taxonomy and Phylogeny.

(August 1976)

William G. Cibula, B.S., John Carroll Univ.,
M.S., John Carroll Univ., Ph.D., University of Mass.

Directed by: Professor Howard E. Bigelow

Nineteen taxa of Hygrophorus subgenus Hygrophorus, section Hygrocybe, sensu Hesler and Smith were examined for pigment composition by chromatography. In one group of species centered about H. miniatus, a group of water-soluble pigments was found which separated well using an acetone/water solvent system and paper chromatography. Two pigments were more abundant than the others in this complex and these two were named provisionally as "rhodohygrocybin" and "flavohygrocybin." Other genera with brightly colored sporophores were also analyzed, but pigments similar to those found in the Hygrophorus miniatus complex were found only in a few species of Amanita.

UV, IR, and NMR spectra are given for flavohygrocybin. Preliminary evidence indicates that this compound is a nitrogen-containing heterocyclic similar to the betalains. Evidence is presented which indicates that rhodohygrocybin also belongs to this group of compounds. The variable color observed in some Hygrocybes is primarily due to variations in the amounts of the magenta rhodohygrocybin and the yellow flavohygrocybin. The

destruction of rhodohygrocybin as the fungus ages leaves flavo-hygrocybin and accounts for the fading to yellow observed in these agarics.

Another group of pigments in other species of Hygrocybe and some species of Mycena are demonstrated to be polyene in nature. These polyenes differ in polarity. The polyenes isolated from H. marginatus are soluble in nonpolar solvents while the polyenes isolated from H. nitidus, Mycena leaiana and M. epipterygia var. cespitosa are freely soluble in water. The primary pigment in M. leaiana is shown by mass spectroscopy to have a molecular weight of 280.

A modification in the taxonomic limits of the section Hygrocybe of Hygrophorus is proposed. Hygrocybe is restricted to those Hygrophori which have rhodohygrocybin and/or flavohygrocybin as well as having both parallel gill trama and clamp connections. These agarics also lack polyene pigments. The species excluded from section Hygrocybe by these criteria are included in section Humidicutis of Hygrophorus providing there are polyene pigments present but no flavohygrocybin and rhodohygrocybin. These species also have interwoven gill trama and usually lack clamp connections on hyphae of the basidiocarp.

A simple spot test to screen agarics for the presence of polyenes is described and discussed.

The problem of a phylogenetic relationship between Cantharellus and Hygrophorus is discussed. Differences in the chemical structures

of the pigments found in the two genera, as well as a reevaluation of other anatomical and morphological criteria, do not indicate that a close relationship exists between these genera. A possible phylogenetic relationship between section Humidicutis of Hygrophorus and the subgenus Glutinipes of Mycena is discussed.

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C H A P T E R I

INTRODUCTION

Hesler and Smith (1963) open their monograph of Hygrophorus with the observation:

> "Color has assumed an importance in the classification of Hygrophorus at the levels of subsection and below since the time of Fries and is reflected in the names used for subgenera and series in the present work. It is thus important to have accurate data on color and a consistent nomenclature for it."

Color has been especially important in defining the species of the section Hygrocybe and these are the focus of this dissertation.

The genus Hygrophorus, a member of the Agaricales (Eumycota), is distinguished primarily by its waxy hymenophore in combination with a white spore deposit. The lamellae are typically thickened, but with sharp edges, and usually subdistant to distant from one another. The basidia of Hygrophorus are typically long and narrow in comparison with other agarics. Basidiospores are smooth and inamyloid, although some investigators may include a few species which have nodulose or amyloid spores.

As might be expected, there are various opinions about the limits of Hygrophorus and the designation of its infrageneric taxa. One modern account may propose generic status for groups which are placed at subgeneric or sectional rank in other studies. For example, Hygrocybe is treated as a genus by Singer (1962), while Hesler and Smith (1963) include this taxon as a section of subgenus Hygrophorus, genus Hygrophorus. Since the genus was first erected by Fries

(1836-1838), there have been a number of accounts, but it is remarkable that the well known species have usually been placed together in groups even though the level of these groups was subject to personal interpretations. A detailed compilation of all works and a discussion of variations is not relevant to the present research problem, but modern treatments of Hygrophorus or Hygrocybe may be found in Kühner and Romagnesi (1953), Singer (1948, 1951, 1958, 1962), Smith and Hesler (1942, 1954), Herink (1958), Hesler and Smith (1963), Donk (1962), Orton (1960), and Orton and Watling (1969). The monograph of North American species by Hesler and Smith (1963) was consulted most frequently in the identification of specimens used for extracting pigments, and this work will serve as a basis. The species are presented in Appendix D according to the classification of Hesler and Smith.

Subgenus Hygrophorus is divided into six sections on anatomical characters by Smith and Hesler:

1. Section Hygroaster, represented by a single species, is distinctive by the nodulose inamyloid spores. (The spores of all other species in the genus are smooth.)
2. Section Amylohygrocybe has only two species, and these are separated on their amyloid spores. (With the exception of Hygrophorus angelesianus of Subgenus Pseudohygrophorus, all other species in the genus have inamyloid spores.)
3. The species of section Hygrotrama are unusual by having the epicutis of the pileus composed of a palisade of enlarged

cells. (Other species have filamentous or gelatinous outer layers to the pileus.)

4. In section Hygrophorus, the species have a hymenophoral trama which is termed "divergent" or "bilateral," that is, the inner lamellar hyphae diverge downward from the pileus context and outward from a central strand of hyphae to give the appearance of an inverted V.
5. Section Camarophyllopsis has species which have a hymenophoral trama composed of intricately interwoven hyphae.
6. Section Hygrocybe has species which have the hyphae parallel to one another, or only slightly interwoven, in the hymenophoral trama.

Section Hygrocybe, of primary concern in the present research, is separated into subsections and series as follows:

1. Subsection Psitticini which has species that have both pileus and stipe with viscid outer surfaces. Three series are recognized. In series Puri, the pileus is sharply conic. In series Inolentes, the pileus is obtuse or depressed and the colors dull -- gray to brown. Series Psittacini also has species with obtuse or depressed pilei but the colors are bright -- red, yellow, blue or green.
2. Subsection Punicei in which the species have a viscid pileus, obtuse to convex in shape, and the stipe is non-viscid. Only the series Punicei is present.

3. Subsection Hygrocybe in which most species have a moist or dry pileus and the stipe is non-viscid. Some species in series Conici have a viscid pileus but all the Conici are separable from the Punicei as well as other Hygrocybes by the conic pileus shape. Other series in subsection Hygrocybe are composed of species with obtuse to convex or depressed pilei. Series Hygrocybe has species with a squamulose pileus, and series Coccinei those with a glabrous pileus.

Purpose and Scope

The purpose of this dissertation is to survey the pigments of certain species of the genus Hygrophorus. Little is known about these pigments, but their composition and distribution can have considerable significance on the taxonomy of the genus and any considerations about the relationships between Hygrophorus and other genera. It is not known if the pigment composition correlates, or not, with anatomical features of basidiocarps, if there are pigments common to certain infrageneric taxa, if the pigments of Hygrophori are the same as known in other organisms, if visible color changes of basidiocarps in the field are due to the loss of particular pigments, if colors which appear the same to the eye are indeed due to certain pigments or mixtures of pigments. The isolation and analysis of pigments can contribute to an answer of such problems.

Species of the section Hygrocybe, genus Hygrophorus, were selected for study for several reasons: in part because of the bright colors present which should be easily adaptable to chromatographic

techniques, in part because there are usually a reasonably large number of species which fruit in the northeast, in part because there has been some work on the bright pigments found in other agarics. Some species in the Cantharellaceae particularly, are believed by some investigators to be related to Hygrophorus in a phylogenetic sequence. The observed differences in the chemical nature of these pigments were related to other anatomical and morphological features of these agarics. As was feasible, species of other genera were sampled to compare their pigments with those of Hygrophorus or to determine if procedures yield results similar to those found by other workers.

History of Research on the Pigments of Agarics and Closely Related Groups

With the possible exception of the alkaloids, much more work has been accomplished on the pigments and other metabolites of molds than with higher basidiomycete pigments. The reasons for this are probably twofold. (1) There has been a definite economic motivation for the elucidation of metabolites produced by the lower fungi (e.g. antibiotic production) and (2) many of the higher basidiomycetes are extremely difficult, if not impossible, to culture with the concomitant production of sporophores in the laboratory. Since the metabolites of interest are often obtainable only from freshly collected sporophores there is a further limitation on their availability for laboratory study. The facilities of a well equipped chemical laboratory may not

be available during a year when fruiting is exceptional, or, when arrangements may have been made for such facilities, sporophore production may be scanty or nonexistent. Additionally, some species are rare or produce a limited number of sporophores regardless of weather conditions.

These problems have been faced by previous researchers on agaric pigments. For example, Birkenshaw and Gourlay (1961) write: "Small quantities of dermocybin were isolated over a period of years in this department, but, owing to the rarity of the fungus, accumulation of sufficient pigment was too slow for degradative studies...." Thus, the data from biochemical studies of agaric pigments have been slow to accumulate and the application to taxonomy has been limited.

Early authors at first limited themselves to the simple observations of color in the field, but later the microscopic aspect of the pigmentation was taken into account. In particular, the studies of Kühner (1934) must be noted where he called attention to significant differences in the pigment topography of agaric sporophores, foretelling differences of a chemical order. From these differences in pigment localization, he expressed an interest in their systematic importance. In particular, he observed that Hygrocybe conica (Fries) Kummer and H. reai (Maire) Lange possess vacuolar pigments.

Turner (1971) and Hegnauer (1962) provides nomenclature and structural formulas on most known fungal metabolites (including basidiomycete pigments and other metabolites). Turner's listings are intended to be comprehensive except for the polyacetylenes, the

depsides, the carotenoids, the ergot alkaloids, the Amanita toxins and the siderochromes. For these groups, Turner only refers to review articles. Hegnauer provides a historical account of early studies of agaric pigments and other metabolites as well as providing a review of the knowledge of the structures of these compounds through 1960. Arpin and Fiasson's (1971) list of known basidiomycete pigments is perhaps the most useful and their schema with the incorporation of more recent studies is followed below. More recently, Eugster (1973) provided a review of many agaric pigments with particular emphasis on those of Russula. The knowledge of pigment types and their distribution within the Agaricales is quite limited. In total, less than 100 species of fleshy fungi have been studied in order to determine pigment types.

Quinonoids.

The quinonone pigments are compounds having a conjugated cyclic diketone structure. p-Benzoquinone, the simplest member of this group, has the structure shown in I. Since quinones are highly conjugated, they are often colored, e.g., p-benzoquinone is yellow in color.

Benzoquinones and benzoquinone derivatives.

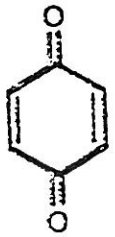
These pigments are extremely abundant among Penicillium and Aspergillus. (Arpin and Fiasson, 1971; Turner, 1971; Miller, 1961), but appear to be much less common among the basidiomycetes. Only two representatives of this type of quinone have been isolated. In

the Agaricales, 2-methyl-5-methoxy benzoquinone (II) has been isolated from Coprinus similis Berkeley and Broome, and from Lentinus degener Kalchbrenner (Anchel et al., 1948).

The pioneering studies on pigments derived from p-diphenyl-benzoquinone was accomplished by Kogl and his colleagues (1924; 1925; 1928). He (1925) was the first to determine the structure of polyporic acid (III) which was isolated from several species of Polyporus. This pigment becomes violet when a sporophore is treated with ammonia.

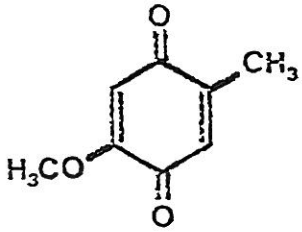
Kogl (1925, 1930), also was first to study the structure of thelephoric acid (IV), but it remained for Gripenberg (1960) to provide the correct structure. More recently, thelephoric acid has been isolated from the cuticle of Suillus grevillei (Klotzch) Singer, (Edwards and Gill, 1973).

Atromentin (V), isolated from Paxillus atromentosus (Batsch ex Fries) Fries, by Kogl and Postowsky (1924), and from Clitocybe subilludens (sic) by Sullivan and Guess (1969) and leucomelone (VI), isolated by Akagi (according to Miller, 1961) from Polyporus leucomelas (Fries) Donk are structurally quite similar. Aurantiacin and dibenzoylleucoaurantiacin, both isolated and identified by Gripenberg (1956, 1958), are related to atromentin, but in aurantiacin the hydroxyl groups on the benzoquinone nucleus are present as benzoate ester groups. In dibenzoylleucoaurantiacin, the center quinone ring is reduced to a hydroquinone and all four hydroxls are benzoylated. These two compounds were isolated from Hydnum aurantiacum Fries



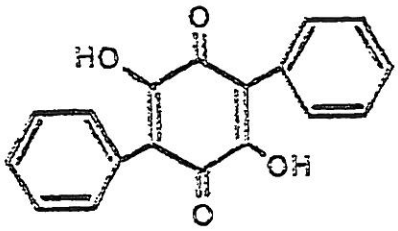
I

p-Benzoquinone



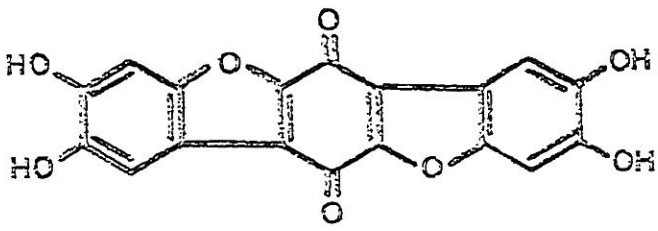
II

2-methyl-5-methoxy-benzoquinone



III

Polyporic acid



IV

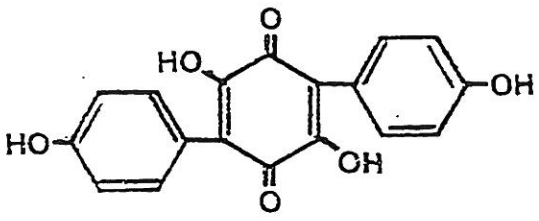
Thelephoric acid

(Hydnellum aurantiacum Batsch ex Fries Karst).

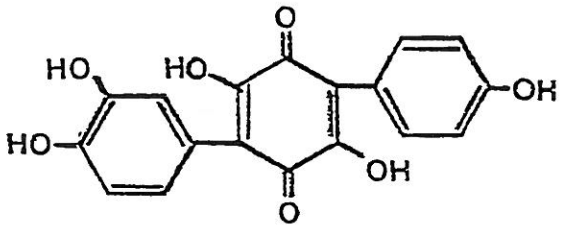
Muscarufin, the red pigment occurring in Amanita muscaria (Fries) S. F. Gray, originally thought to be a p-diphenylbenzoquinone derivative from the studies of Kogl and Erxleben (1930), is now known to be quite different in structure. (Besl et al., 1975; Dopp and Musso, 1974; Eugster, 1973; Turner, 1971; Eugster, 1969; Talbot and Vining, 1963; Nilsson and Norin, 1960). This pigment will be discussed later.

Although not directly a p-diphenylbenzoquinone derivative, it will be well to consider involutin (VII), which is not a pigment but may be a precursor, produced by Paxillus involutus (Batsch ex Fries) Fries (Edwards et al., 1967). This compound can be interrelated chemically (and presumably in nature) by the oxidation of the quinones to give the lactones which can then give rise to the cyclopentane derivatives. Figure 1 gives a possible scheme for the chemical conversion of benzoquinones to lactones and then to cyclopentanones, Turner (1971).

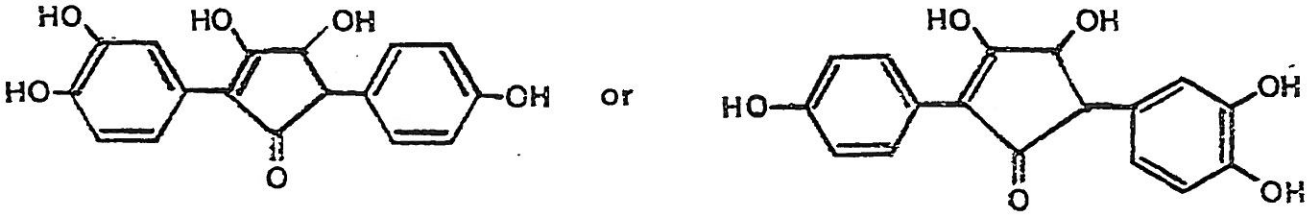
Related to these structures are: variegatic acid lactone (VIII a) isolated from Suillus variegatus (SW. ex Fries) Kuntze, (Edwards and Elsworthy, 1967; Beaumont et al. (1968), while variegatic acid (XXV) has been isolated from S. bovinus (L. ex Fries) Kuntze; xerocomic acid lactone (VIII b) isolated from Xerocomus chrysenteron (Bull. ex St.-Amans) Quel. (Steglich et al., 1968) and also from Gomphidius glutinosus (Shaeffer ex Fries) Fries (Steglich et al., 1969); and gomphidic acid lactone (VIII c), also from Gomphidius glutinosus (Steglich et al., 1969).



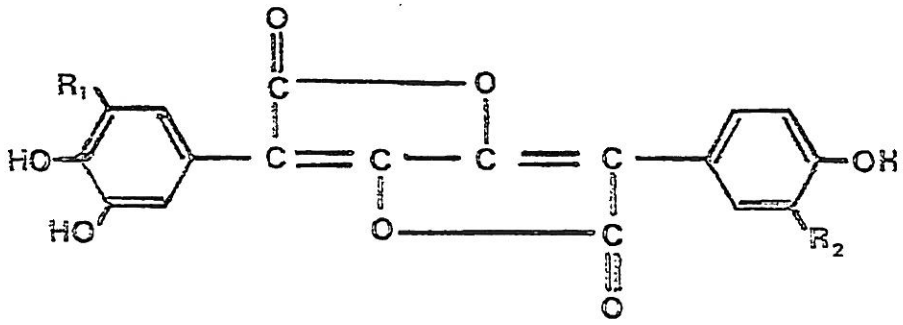
V Atromentin



VI Leucomelone



VII Involutin



VIII

	R_1	R_2	Structure Name
a	H	OH	variegatic acid lactone
b	H	H	xerocomic acid lactone
c	OH	H	gomphidic acid lactone

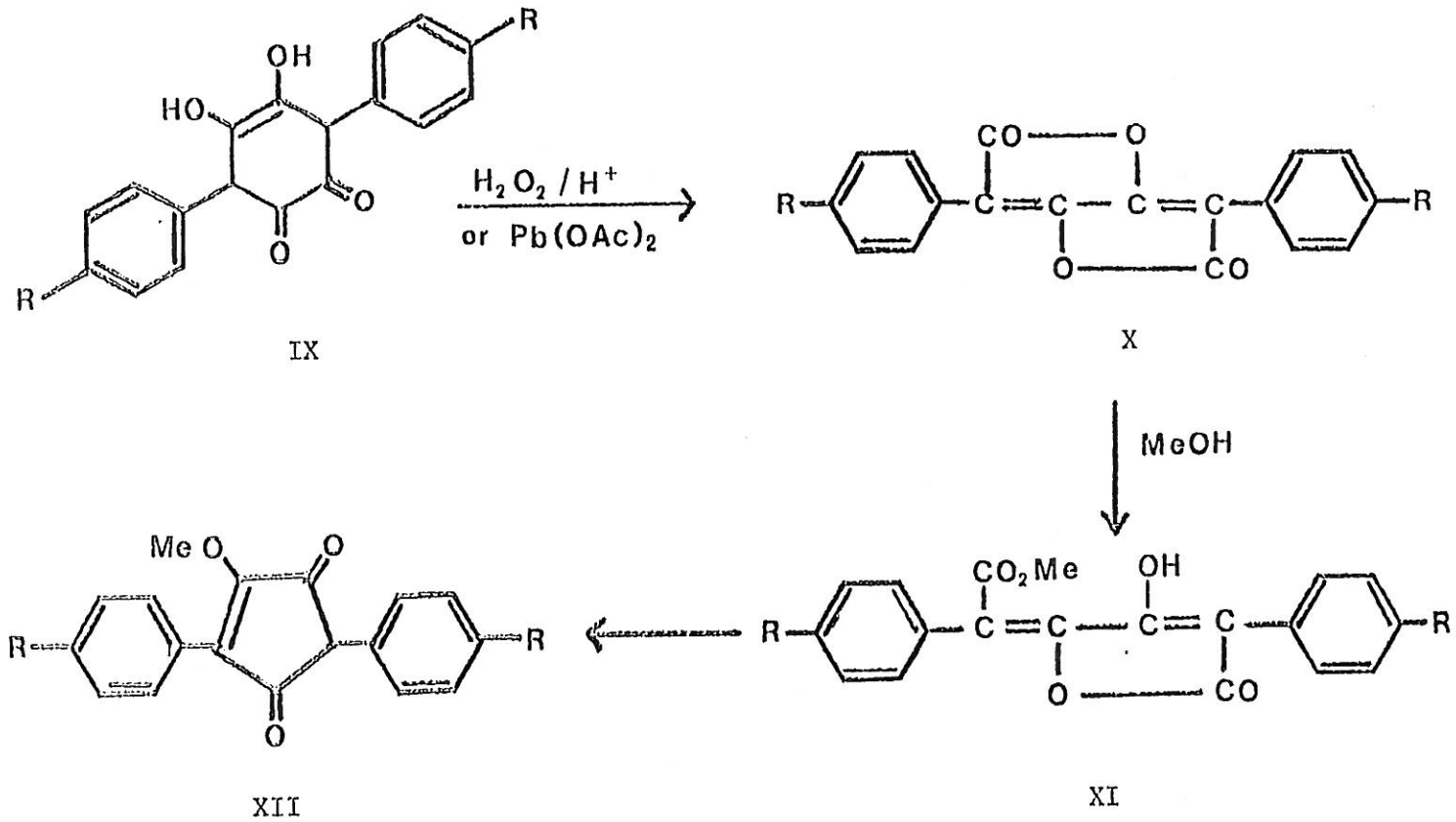


Figure 1

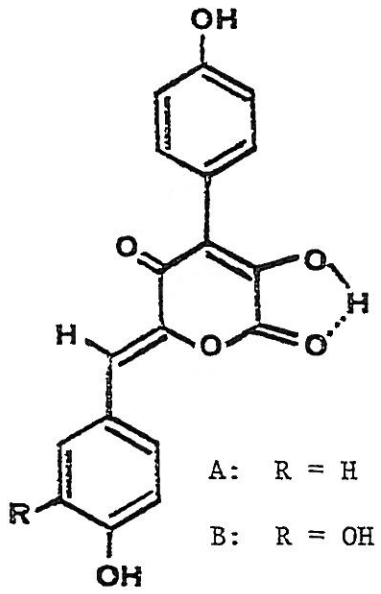
Possible scheme for the chemical conversion of benzoquinones to lactones and to cyclopentanones.

Grevelline A, B and C have been isolated and identified by Steglich, Besl and Prox (1972). The structures proposed for these yellow pigments are shown in XIII and XIV. They were isolated from Suillus grevillei (Klotzsch) Singer and were also demonstrated to be present in Suillus tridentinus (Bresadola) Singer. Very recently, Bresinsky, Best and Steglich (1974) reported the isolation of gyroporin (XVI) and atromentic acid (XV) from cultures of Leccinum aurantiacum. Eugster (1973) reports that the pink coloration found in Suillus bovinus (L. ex Fries) Kuntze (Linnaeus) Quelet, is due to the combination of the orange colored bovinon-4 (XVIII), the yellow orange amitenon (XVII) as well as atromentin (V). He also reports that bovinon-3, a benzoquinone closely related to bovinon-4 and also containing an isoprenic sidechain occurs in Chroogomphus rutilans (Fries) O. K. Miller.

A dilactone, variegatorubin (XXVI) and variegatic acid (XXV, lactone, VIII) are also found in Suillus bovinus, Boletus luridus Fries, and Phylloporus rhodoxanthus (Schweinitz) Bresadola. This pigment is responsible for the rose to reddish colored stipe (Eugster, 1973).

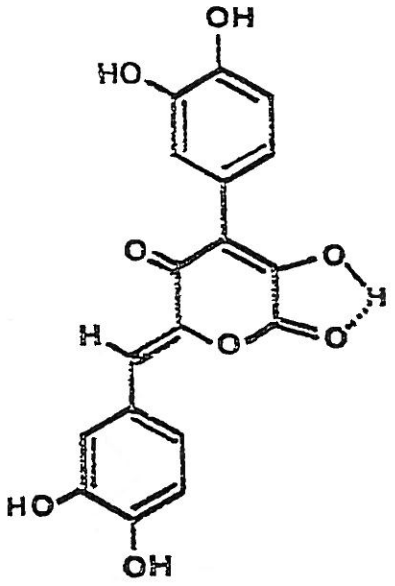
Naphthoquinone derivatives.

These compounds are found extensively in the lower fungi (see Turner, 1971 for a listing), but are little known in the basidiomycetes. However 6-methyl-1,4-naphthoquinone (XIX) has been isolated from Marasmius gramineum Lib. by Bendz (1948).



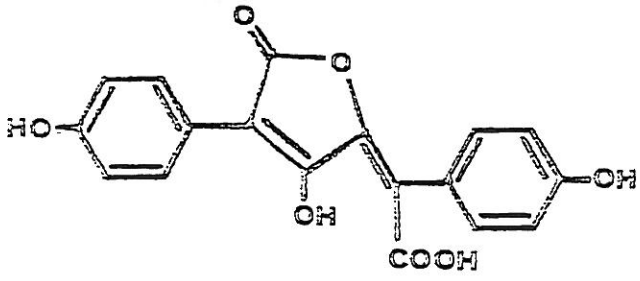
XIII

Grevilline A & B



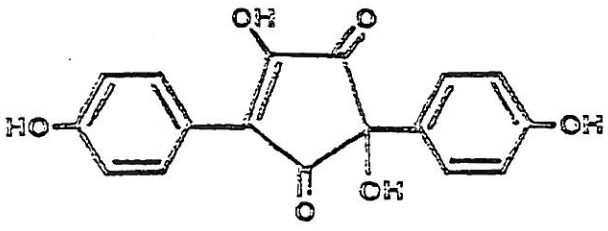
XIV

Grevilline C



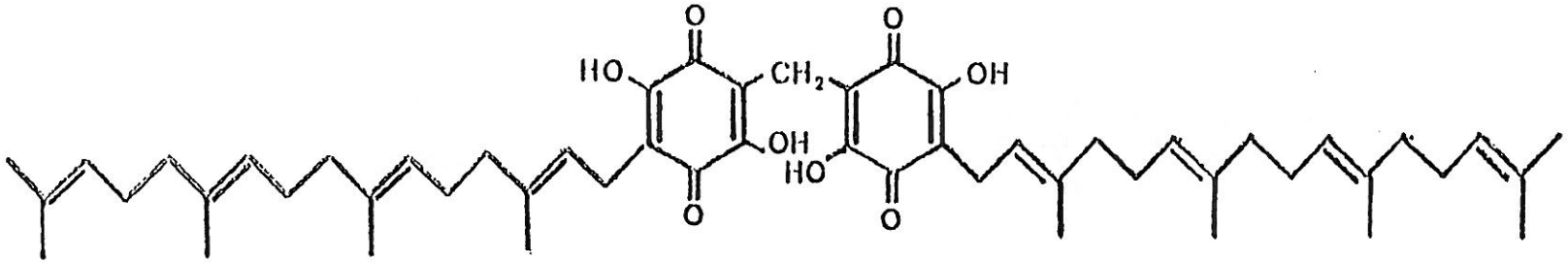
XV

Atromentic acid



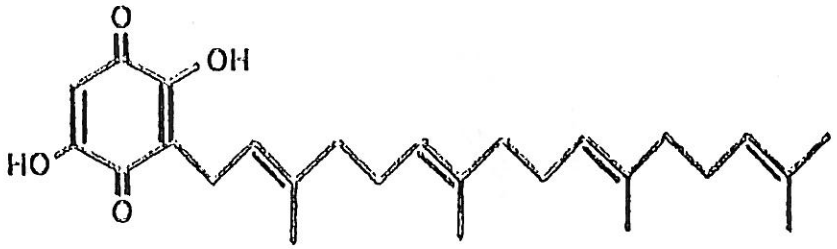
XVI

Gyroporin



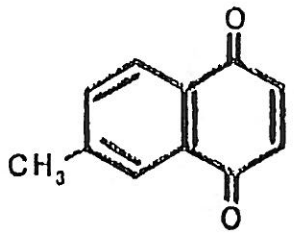
XVII

Amitenone



XVIII

Bovinone-4



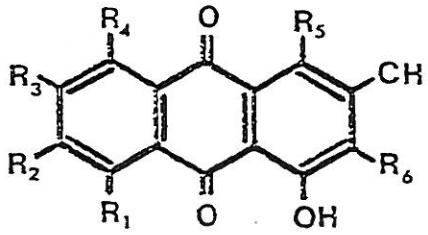
XIX

6-Methyl-1, 4-naphthoquinone

Anthraquinone derivatives.

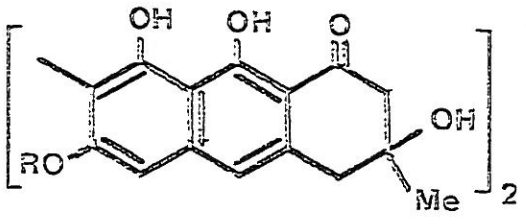
Again, these pigments are widespread among the lower fungi, being quite prevalent in various Penicillia. Several are found in the Agaricales. Emodin (XXa) and dermocybin (XXb) have been isolated by Kögl and Postowsky (1925a), as well as by Steglich and Austel (1966). The latter authors report the presence of dermoglaucin (XXc) in the same paper. All of these pigments have been isolated from Cortinarius sanguineus (Wulf. ex Fries) Fries. More recently, Steglich et al. (1969) have isolated the above pigments along with physicon (XXd), endocrocin (XXe), and four new anthraquinone carboxylic acids (XXf to XXi), from both Cortinarius sanguineus and C. semisanguineus Fries. Additionally, Gabriel (1965), has found these pigments along with a number of structurally unidentified anthraquininilic isomers (some, perhaps those above identified by Steglich) in other Cortinarii. Emodin was found to be present also in C. malicorius Fries.

In 1972, Steglich and co-workers reported on flavomannin-6, 6'-dimethyl ether (XXI) from Dermocybe cinnamomeolutea (Orton) Moser, D. uliginosa (Berkeley) Moser and D. paiustris var. sphagneti (Orton) Moser. These authors also demonstrate that a racemate of this same pigment is responsible for the coloration of Tricholoma flavovirens (Fries) Lundell. Earlier, 7, 7' biphysicon (Gluchoff et al. 1972) had been reported as a pigment in this same agaric but Steglich et al. (1972) state that this compound is an artifact formed during the isolation procedure from a flavomannin type precursor.



XX a-i

	R_1	R_2	R_3	R_4	R_5	R_6	NAME
a	OH	H	OH	H	H	H	emodin
b	OH	OH	OCH_3	OH	H	H	dermocybin
c	OH	OH	OCH_3	H	H	H	dermoglaucin
d	OH	H	OCH_3	H	H	H	physicon
e	OH	H	OH	H	H	COOH	endocrocin
f	OCH_3	H	OH	H	H	COOH	dermolutein
g	OCH_3	H	OH	H	OH	COOH	dermorubin
h	OCH_3	H	OH	Cl	H	COOH	5-chlorodermolutein
i	OCH_3	H	OH	Cl	OH	COOH	5-chlorodermorubin



XXI

a) R = Me, flavomannin-6,6'-dimethyl ether

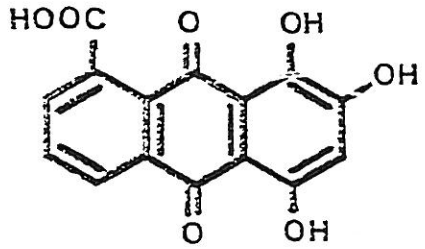
b) R = H, flavomannin

Mme. Gabriel's study of these pigments in Cortinarius is an excellent example of the application of a study on the chemical nature of pigmentation to taxonomic problems. She observed two anthraquinone derivatives which were common to all species, save one, of the subgenus Dermocybe, while within this subgenus several anthraquinones were specific only for a particular section. She found the section Sanguinei to be characterized by two specific pigments which were absent elsewhere, while the section Cinnamomei had some species with anthranols as well as three other anthraquinones not found in section Sanguinei. Within the subgenus Hydrocybe section Miniatopodes, there were four anthraquinolic vacuolar pigments which are responsible for the characteristic colors of this section, e.g., C. bulliardi Fries. Cortinarius cinnabarinus Fries, has been placed in the subgenus Dermocybe, however C. cinnabarinus has a hygrophanous pileus and Kühner has stressed the great resemblance in the color of the stipes to that of C. bulliardi. In her studies, Gabriel found that these two species possessed the same four pigments and that C. cinnabarinus lacked the pigments found in other species of the subgenus Dermocybe. In this sense, "...the resemblance between the pigments of this species (C. cinnabarinus) and those of bulliardi is striking and would seemingly allow us to place cinnabarinus with bulliardi considering it (cinnabarinus) a Hydrocybe of the section Miniatopoda in which the red pigmentation pervades the whole carpophore, rather than being limited to the base of the stipe." (Translation, Gabriel,

1965).

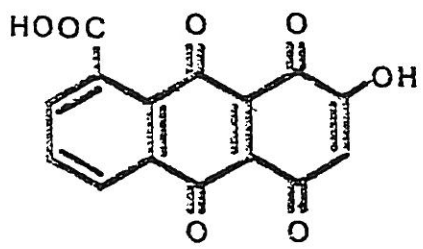
In the Boletaceae, another anthraquinone derivative, boletol, (XXII), has been thought to be responsible for the bluing reaction of many boletes. It had been thought that the oxidation of boletol to boletoquinone (XXIII), produced the blue coloration. More recently, the structure of boletol has been questioned and it has been demonstrated that the bluing reaction in the Boletaceae is caused by oxidation of tetraonic, variegatic and xerocomic acids (VIIIa, b) rather than boletol and isoboletol (Edwards et al., 1967; Steglich et al., 1968). One of these blue oxidation products is shown in XXVII. This is the deep blue anion which results from the oxidation of variegatic acid.

A review of the distribution of "boletol" (formerly believed to be responsible for bluing in a number of boletes) is given by Gabriel (1965). She discusses the distribution and taxonomic significance of this compound in comparison with more classical concepts. As an example, the species of Boletus sensu Singer (subfamily Boletideae) all contained "boletol" (now known to be variegatic acid derivatives). However, in B. edulis Bulliard ex Fries, the context is white and "boletol" is found only in the tubes at maturity. In Leccinum, "boletol" is found in some as in L. aurantiacum. Both Leccinum and Boletus react with phenoloxidase. In contrast to these, Tylopilus felleus (Bulliard ex Fries) Karste (white context) does not contain "boletol".



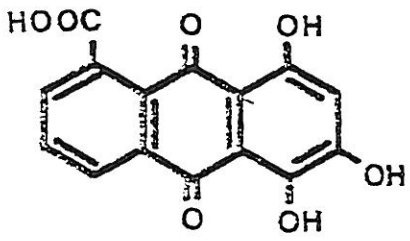
XXII

Boletol



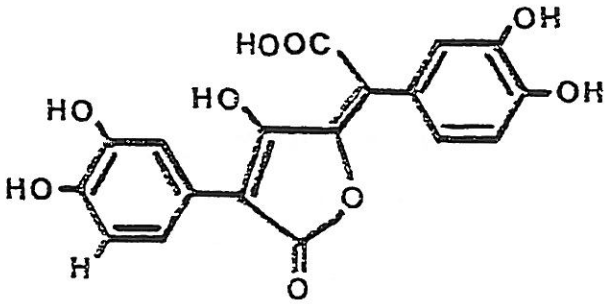
XXIII

Boletolquinone



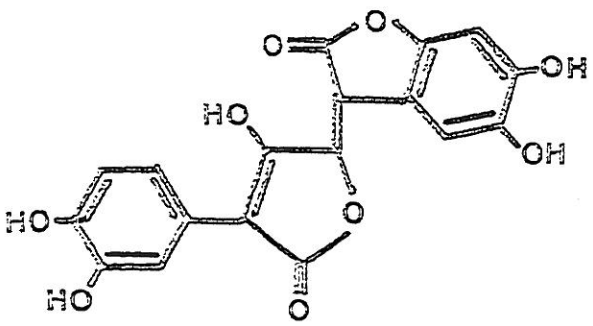
XXIV

Isoboletol



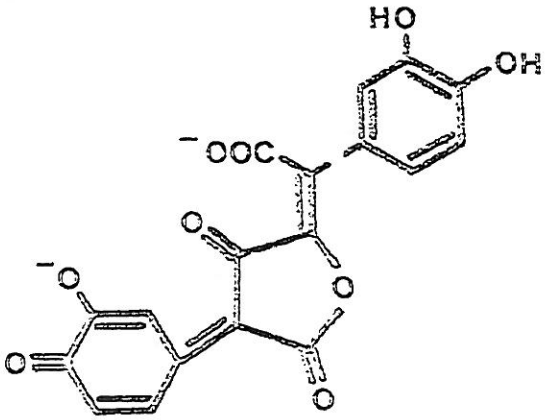
XXV

Variegatic acid



XXVI

Variegatorubin



XXVII

Among taxa suspected of being related to the Boletaceae, Phylloporus rhodoxanthus (Schweinitz) Bresadola does contain "boletol". In contrast to this, members of the Gomphidiaceae do not contain "boletol" but in all species examined the presence of "pseudoboletol" has been definitely established. The Paxillaceae contain neither "boletol" or "pseudoboletol" and Gabriel observes that this is consistent with classical concepts. This family has been considered farther removed from the Boletaceae than any of the preceeding as the spores are not fusiform.

Aliphatic Polyenes.

The existence in Basidiomycetes of non-isoprenic polyenes as pigment types was demonstrated when corticrocin (XXVIII), a polyene dicarboxylic acid was isolated from Corticium croceum Bresadola by Erdtman (1948). Later another polyene pigment, cortisalin (XXIX), was isolated from Corticium salicinum. These species are not very closely related to agarics, but similar pigments were found in Hygrophorus.

Carotenoids.

The true carotenoids, which occur throughout the plant kingdom, are tetraterpenoids--yellow to red, generally fat soluble pigments. Many fungi do not synthesize carotenoids, but certain generalities may be given regarding their distribution in fungi (Goodwin, 1965).

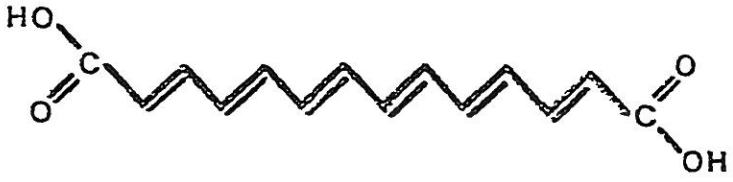
a) β-carotene is not universally present, but is well distributed in the Mucorales (Hesseltine, 1961).

b) The presence of the major characteristic xanthophylls of higher plants has never been unequivocally established and such minor compounds as zeaxanthin and cryptoxanthin occur only occasionally.

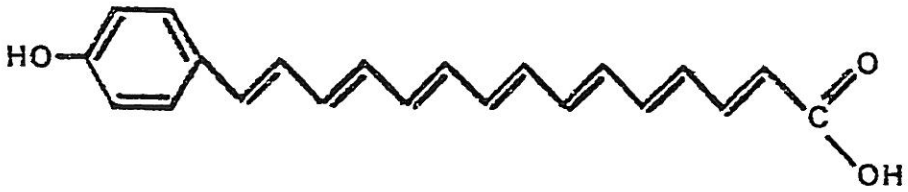
c) Characteristic fungal carotenoids are frequently acidic, for example, torularhoden (Ruegg et al., 1958) and neurosporxanthin (Zalokar, 1957).

Fiasson (1968), notes that methoxyl groups such as those present in some of the carotenoids of many of the purple photosynthetic bacteria (e.g. spirilloxanthin) and the epoxides which are so frequent in the xanthophylls of green plants (e.g., violaxanthin) appear to be unknown among the fungi. This author further observes that our knowledge of carotenoids in fungi is at best very scanty; of some 100,000 species of fungi described, only about a hundred have been examined for the presence or absence of carotenoids. Far fewer agarics have been examined. As a prelude to his studies, Fiasson presents a table listing all previous studies of carotenoids in all groups of fungi including also studies made with representatives of the Myxomycetes. This reference, along with Turner (1971), gives a fairly complete review of previous studies on fungal carotenoids up until very recent times.

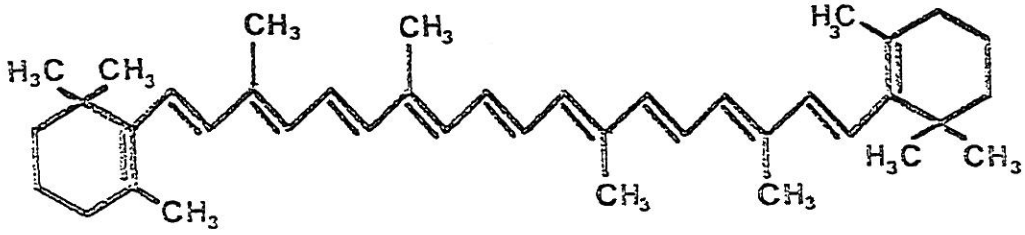
The pioneering studies on carotenoids in agarics were the investigations of Zopf (1890), Kohl (1902) and von Wisselingh (1915). Zopf determined if the pigments present in various agarics were "lipochrom" or not. Later, Willstaedt (1937) examined the



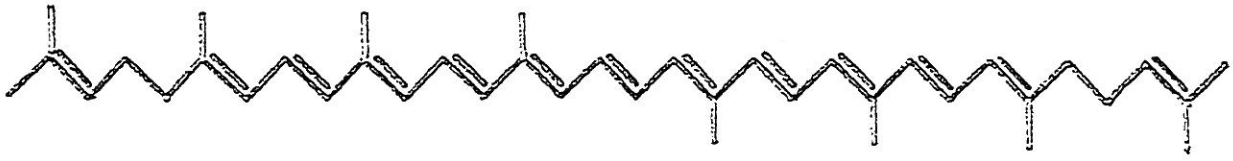
XXVIII Corticrocin



XXIX Cortisalin



XXX β-Carotene

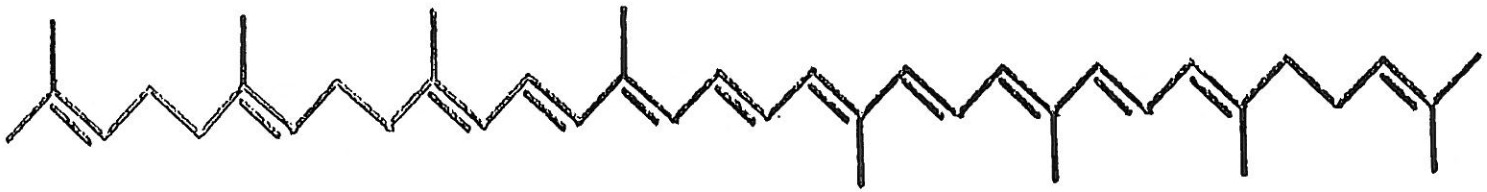


XXXI Lycopene

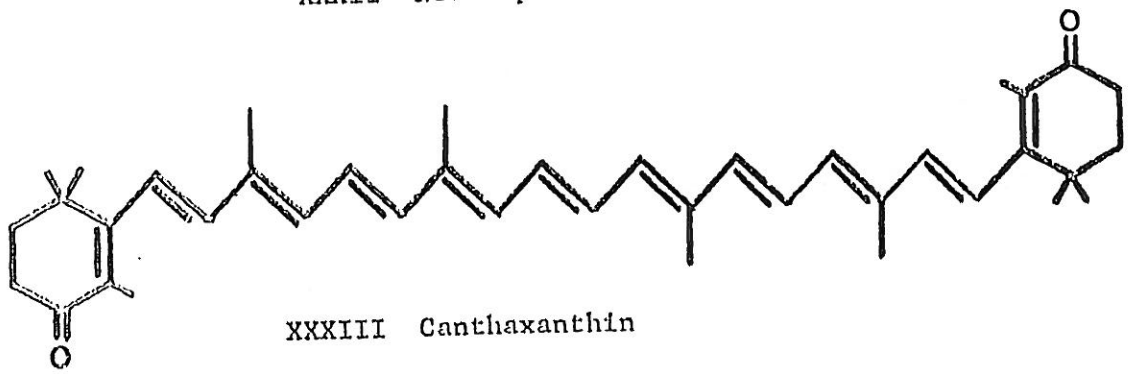
pigments in Cantharellus cibarius Fries, Cantharellus lutescens (Persoon) ex Fries and Cantharellus infundibiliformis Fries. In the first species he found primarily β-carotene (XXX) but carotene and lycopene (XXXI) were also present. In both C. lutescens and C. infundibiliformis the major component was an unknown carotenoid along with appreciable amounts of lycopene and possible β-carotene. Turian (1960) determined that the major pigment in C. infundibiliformis was neurosporene (XXXII) while Fiasson and Arpin's (1967) study determined that neurosporene was indeed present and that phytofluene, carotene and a xanthophyll were also present in smaller quantities.

The rare carotenoid canthaxanthin (XXXIII) was first isolated and identified by Haxo (1950) from Cantharellus cinnabarinus Schweinitz. This pigment is responsible for the cinnabar-red coloration. Haxo also found five other carotenoid pigments, but these were present in much smaller amounts.

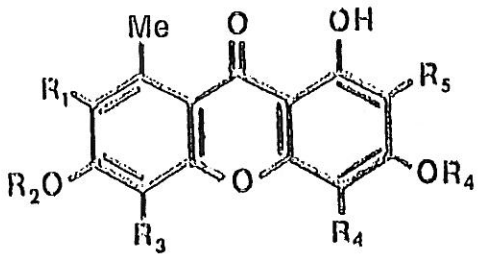
Arpin (1966) extracted the pigments from Clitocybe venustissima (Fries) Saccardo, and through the use of column chromatography with alumina, determined that β-carotene and γ-carotene account for more than 80% of the total pigment. He also examined Hygrophoropsis aurantiaca (Wulfen ex Fries) R. Maire, but found that the pigments were not soluble in petroleum ether and were entirely different from those C. venustissima. On this basis, Arpin discounts a close relationship between the two species, noting that the similarity of orange colors is extremely superficial.



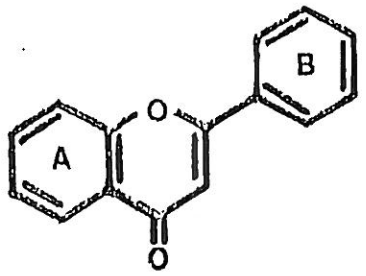
XXXII Neurosporene



XXXIII Canthaxanthin



XXXIV Xanthone



XXXV Flavone

Fiasson and Bouchez (1968) determined that β-carotene was the principal pigment in Omphalina chrysophylla (Fries) Murrill with -carotene, torulene and lycopene also being present in smaller amounts. These authors believe that the presence of these pigments indicate that there is a phylogenetic relationship between this fungus and the Cantharellaceae. Fiasson et al. (1970) undertook a comparative study of the carotenoids present in certain species of the Cantharellaceae and Clavariaceae where he found carotenoids in only two species of ten Clavarias studied, and among the cantharelloid fungi, these pigments were only found in those species centered about the genus Cantharellus in its narrowest interpretation.

Carotenoids of a polar nature have been isolated from fungi of the nematophagous series (Valadon et al., 1963). Such carotenoids are not frequently encountered and their polar nature gives them properties not usually associated with this class of pigment.

Xanthones.

Xanthones are pigments of the structure shown in XXXIV where R can be hydrogen, methyl or chlorine.

In a study of the pigments of Cortinarius, (Gabriel, 1965), reports the presence of a xanthone type pigment in species studied that are found in the section Olivaceoaurati Lang, subgenus Cortinarius (=Inoloma Fr.). The species examined were: C. cotoneus Fr. (sensu Quelet), C. venetus Fr., and C. melanotus Kalchbr. The

pigment exists in glycosidic form. UV absorption spectra yielded four absorption maxima. This xanthone is not identified, but differs from those already known in that it is hydroxylated in positions 1 or 8. Addition of silver nitrate causes the pigment to become intensely yellow. She regarded the demonstration of the nature of this pigment to be most important since: a) hydroxy xanthones were not known except in the lower fungi, Roberts (1961); Turner (1971) , and b) it had not been established with any certainty that glycosidic pigments exist in both the lower and higher fungi.

Flavonoids.

The term flavonoid was applied first about twenty years ago by Geissman and Hinreiner (according to Goodwin, 1965) to encompass compounds whose structure is based on that of flavone (XXXV). These compounds consist of two benzene rings, A and B, jointed together by a three-carbon link to form a γ-pyrone ring. The various classes of flavonoid compounds differ from each other only by the state of this 3-C link. The anthocyanin pigments are one of these compounds.

Harborne (1965) notes that water soluble pigments of the flavonoid group are very widely distributed in nature, and that these pigments are characteristic only of the higher plants and are virtually absent from lower plants. One of the large classes of flavonoids, the anthocyanins, appear to be entirely absent from fungi. However, Robinson and Robinson (1934), do report that the pink coloration in the lamellae of some Agaricus species (including the

cultivated mushroom) are anthocyanin pigments. In contrast to this, there is more recent speculation that this coloration may be due to dopachrome with polymerization of the dopachrome derivative, 5, 6-dihydroxyindole, yielding the brown melanin type pigments observed in maturity in Agaricus (Fox et al., 1960).

Sesquiterpene derivatives.

Sesquiterpene derivatives are C_{15} compounds of considerable variation in structure. Only a few are sufficiently unsaturated to be pigments. Three closely related sesquiterpene pigments have been isolated from Lactarius deliciosus (Fr.) S. F. Gray, namely lactaroviolin (XXXVI) by Heilbronner and Schmid (1954); lactarazulene (XXXVII), by Sorm et al. (1953) and lactarofulvene (XXXVIII) by Bertelli and Crabtree (1958).

Pteridine derivatives.

Although it is now known that the Russula pigments are pteridine derivatives, attempts to characterize the pigments of brightly colored species of this genus dates back to the investigations of von Schroeter (1875) and Phipson (1882). Phipson examined the red extract from the pilei of Agaricus ruber (Russula sanguinea?, Watson, 1966).[1]

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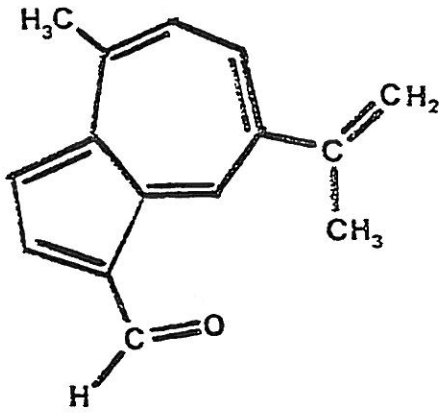
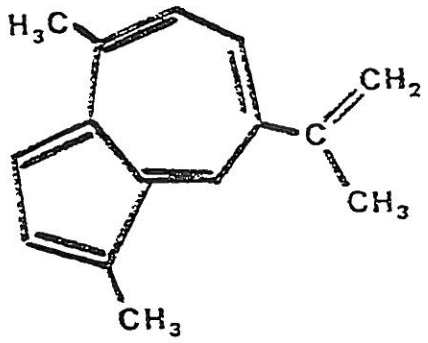
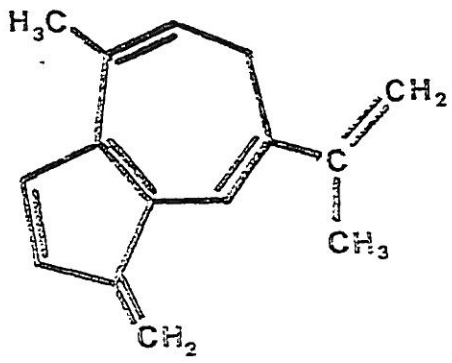
[1] Watson (1966) believed Phipson's study to be an extractive from Russula sanguinea, while Zopf (1890), discussed the study of Phipson's extractive separate from both his discussions of Russula pigments and the pigments of Amanita muscaria. It is not clear where Zopf would have classified the agaric which Phipson has studied.



XXXVI Lactaroviolin



XXXVII Lactarazulene



XXXVIII Lactarofulvene

He named the pigments ruberine, observing also, that the extract was fluorescent. In 1890, Zopf summarized these studies and noted that the pigment is not extractable with absolute alcohol, diethyl ether, carbon disulfide, chloroform or benzene. The aqueous solution fluoresces with a blue to blue-green color.

After these early studies, there is a lapse of interest in these pigments, and Pastac (1942), does not mention these studies in his monograph on fungal pigments.

More recently Josserand and Nétien (1938, 1939), observed during a study of the fluorescent aspect of various agarics under a long wavelength ultraviolet lamp, that the species of Russula which were examined were brightly fluorescent. In contrast, the Lactarii examined were dull. This observation led these authors to conclude that this difference indicated a chemical division between the two genera which verifies the traditional separation of these genera on morphological criteria. The ultraviolet fluorescence of Russula sporophores was also reported by Deysson (1958).

Kühner (1934) noted that the pigments of Russula are of a vacuolar nature in contrast to Lactarius where the pigment is usually membranal or intercellular. Furthermore, the pigments in Lactarius which have been studied are of a lipophilic nature. For example, Willstaedt (1935, 1936, 1946a, b) first isolated lactarazulene and lactaroviolin from L. deliciosus (Fries) S. F. Gray, and these are both lipophilic. In contrast, the pigments of many Russula species are soluble in more polar solvents, indicating a possible

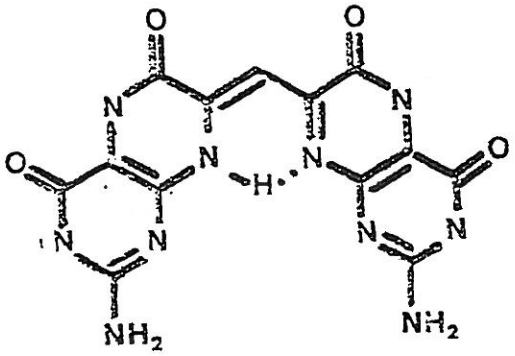
difference in chemical architecture. In 1955, Balenovic et al., examined the pigments of Russula emetica (Shaeff. ex Fr.) S. F. Gray isolating the major red component which they named "russularhodin." Additionally paper chromatography and electrophoretic techniques were used to examine other pigment components among ten species examined. These authors found some pigmented and fluorescent compounds which were common to all ten species while other compounds were specific for only a given species. Although Balenovic et al. did not group the ten species they examined, they did suggest that chromatography and electrophoresis could be powerful taxonomic tools to discern interrelationships between species. Pauline Watson (1966), undertook an investigation of 26 "representative" species of Russula in which the extracts were chromatographed on silica gel TLC plates. She observed at least three red, seven yellow and three blue pigments. From this, she was able to place the species studied into four groups according to the major pigments separated. These were unnamed but identified by their color and R_f value. In 1970, Frauenfelder published his dissertation where he describes his study of the Russula pigments. He provided ultraviolet, visible, infrared and NMR spectra for a number of purified fractions which he isolated and studied. He suspected that these pigments were complex nitrogen-containing heterocyclics which were possibly related to the pteridins.

The first success in characterizing the chemical nature of the Russula pigments must be credited to Eugster and Frauenfelder (1970). They remark that recent studies with paper electrophoresis (Balenovic

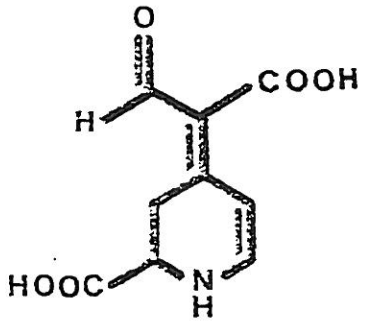
et al., 1955), paper chromatography (Bonnet, 1959; Gluchoff, 1969) TLC on silica gel (Watson, 1966) and column chromatography with cellulose have indicated that the Russula pigments are mixtures which are extremely difficult to separate. In fact, a preparation of individual pigments in pure condition was not obtained by any of these earlier authors. By use of isoelectric focusing using a pH and sucrose gradient, Eugster achieved a resolution of many more components from Russula sardonia Fries em. Romagnesi than he could achieve on a Sephadex F-25 column. Purification of the zone at pH 7.29 which was the major component, yielded what Eugster believes to be a riboside derivative of a dimeric pteridin. He named this Russupteridin-s III and stated the structure is similar to that shown in XXXIX for rhodopterin. This is the first proof of a dimeric pteridin and a pteridin riboside in a plant, although similar compounds have long been known as the coloring matter in some Lepidoptera. It is especially noteworthy that a rich source of pteridin derivatives is now available.

Amanita muscaria pigments.

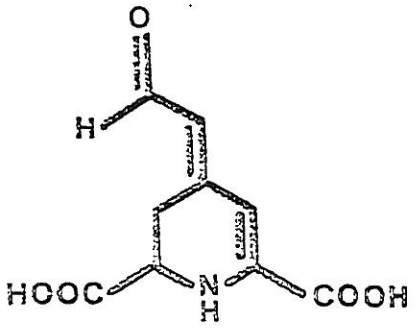
The early studies of these pigments undertaken by von Schroeter (1875), Weiss (1885), Bachmann (1886) and Zopf (1890) emphasized the high solubility in water and the very limited solubility in most organic solvents as well as giving some rudimentary spectral data and fluorescent aspects. Zellner (1906), summarizes the knowledge of the Amanita muscaria pigments as it existed shortly after the



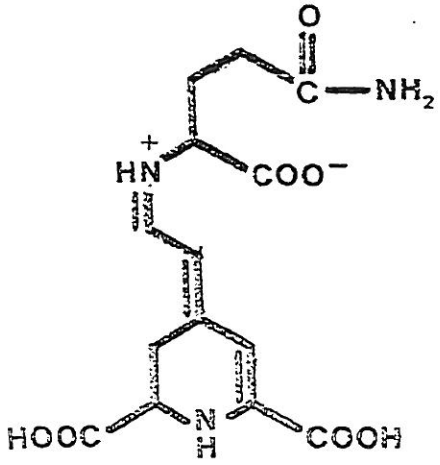
XXXIX Rhodopterin



XL "Muscaflavin"



XLI Betalamic acid



XLII Vulgaxanthin

turn of the century. Zellner found that the pigments were localized in the cellular fluid of the pileus cuticle and are removed from the cuticle by only water or alcohol. The pigments in solution do not exhibit indicator qualities, but concentrated solutions exhibit a weak green fluorescence. The pigments are precipitated by the addition of lead acetate and the precipitate can be reduced by the addition of hydrogen sulfide. He also studied various colorless compounds which also were present.

In 1930, Kögl and Erxleben reported the isolation of the red pigment from the pileus of the Fliegenpilz (A. muscaria) and they named it muscarufin. This pigment was isolated by extraction with alcohol and precipitated as the silver salt, then regenerated with methanolic hydrochloric acid. They suggested that muscarufin was a terphenyl quinone, (see Thompson, 1957, structure 77, p 34), and from 1930 to 1960 the structure was not questioned. Nilsson and Norin (1960) attempted to synthesize compounds related to muscarufin, but during their studies evidence accumulated which cast doubt on the correctness of the structure of muscarufin as proposed by Kögl and Erxleben. In 1963, Talbot and Vining isolated three water soluble pigments from sporophores collected near Saskatoon, Saskatchewan. Recognizing that the pilei were generally lighter in color than in European specimens, these authors found that the properties of the crude red pigment corresponded very well with those reported by Zellner for his European collections. However, there was no evidence for the presence of muscarufin or its glycoside precursor

in the Canadian specimens. The pigments of A. muscaria were found to be quite unstable in light, air, acid or alkali, and could not be isolated in a pure state, but the limited results suggested that these pigments might belong to the pterin or flavin class of compounds. Eugster (1969) observes that the silver salt precipitation with consequent regeneration of the pigment upon addition of hydrochloric acid does not give the results reported by Kögl and Erxleben. With addition of hydrochloric acid, only degradation products of undefined structure result. He seriously doubts the existence of a pigment with Kögl's structure for muscarufin. Eugster (1973) writes that it is with great surprise to report that the pigments of Amanita muscaria and A. caesarea are betalains. These are pigment types which were first isolated from the red beet (Beta vulgaris var. rubra). Amanita muscaria contains one yellow, seven orange, one red-brown and one magenta pigments all present in a mixture which is extremely difficult to purify (separate). He further remarks that the bizarre structure proposed earlier by Kögl and Erxleben can hardly exist.

Döpp, Grob and Musso (1971) isolated four pigments from A. muscaria, namely muscapurpurin (max 540nm), muscarubin (max 478) and muscaflavin (max 420). These were later demonstrated to belong to the same chromophoric system as the betalains (Döpp & Musso, 1973a, b). Muscaflavin (XL) is believed to be an isomer of betalamic acid (XLI). Muscaaurin has a structure similar to that for vulgaxanthin I (XLII) while muscapurpurin is quite similar to betanidin (XLIII).

More recent investigations show the nitrogeneous heterocyclic to be a seven membered ring and both Besl et al. (1975) and von Ardenne et al. (1974) give structure XLIV for muscaflavin.

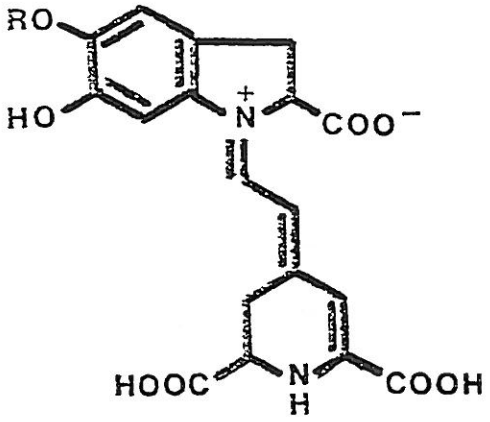
In addition to these most unusual pigment types, A. muscaria is relatively rich in vanadium and contains up to 120 ppm vanadium per gm of dry weight tissue. These findings were reported by Bayer and Kneifel (1972) who isolated amavadine from this agaric. This compound is blue and does not possess a melting point. A later paper (Kneifel and Bayer, 1973) gives a tentative structure (XLV) for amavadine.

The reviews above illustrate the diversity of pigment types to be found in different agaric groups.

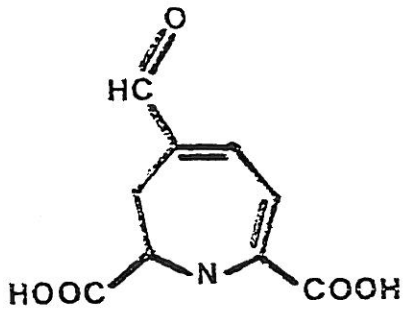
Pigments in Hygrophorus.

The earliest studies of these pigments are those of Bachman (1886). Zopf (1890) summarized these as well as making his own observations. He believed that the difference in color between the yellow to orange Hygrophorii such as H. conicus Scopoli, and scarlet ones, such as H. coccineus Schaeffer or H. puniceus Fries, was due to differing concentrations of a single pigment.

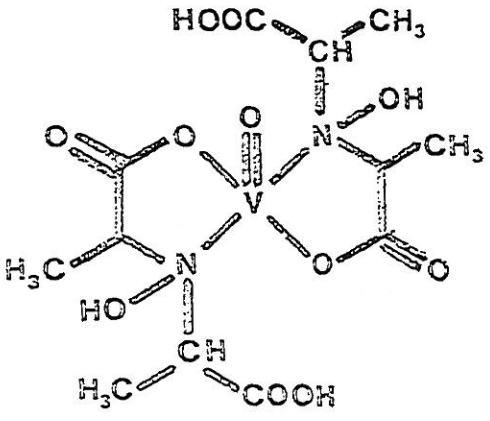
Zopf found that extraction is best achieved by soaking the pileus in water since the pigment is not extractable in absolute alcohol, 96% alcoholic solution, or benzene. The yellow liquid which results from extraction and evaporation yields a saffron-yellow gummy substance. Addition of aqueous sulfuric acid to this causes a



XLIII Betanidin



XLIV Muscaflavin



XLV Amavadine

change to reddish. Addition of aqueous sodium hydroxide produces a pale yellow color which finally becomes completely colorless. The addition of lead acetate causes a blood-red precipitate to form which does not dissolve entirely in acetic acid but is completely dissolved in sulfuric acid. The pigment is characterized by its marked absorption in the blue end of the spectrum. Zopf makes the further observation that there appears to be a great similarity of this pigment to the Russula pigments, but the major differences are in the reactions in alkali and sulfuric acid.

Fiasson (1968) in his survey of carotenoid pigments in basidiomycetes, examined the pigment in H. puniceus Fries. He employed acetone for the primary extraction. Petroleum ether (b. p. 40-60°C) was then added to the acetone pigment mixture along with sufficient water to allow the two phases to separate. After shaking, the carotenoids if present would be found in the upper phase. The crude acetone extract exhibited a very broad and rounded absorption peak around 416 nm with slight shoulders at 455 and 480 nm. Fiasson found that the pigment is strictly hypophasic (not a carotenoid), with the epiphase (pet. ether) showing only the absorption peaks of ergosterol.

During 1967, Maureen Brett Esborn (personal communication) studied the separation of Hygrophorus (sec. Hygrocybe) pigments under the author's guidance as a science project for the Northeastern Ohio Science Fair. She used Eastman TLC sheets and found that the extracts from Hygrophorus flavescens and H. cantharellus yielded several separate

bands using an acetone/H_2O solvent as the developer.

More recently, Sulya (1971) studied the pigments in Hygrophorus marchii Bresadola. The specimens were first frozen in a refrigerator-freezer and then later freeze-dried. The fruiting bodies were pulverized with quartz sand and then extracted with a 47.5% aqueous ethanol solution or a 75% aqueous methanol solution. When methanol was used for extraction, a cream colored precipitate settled out. Spectrophotometry of the crude extract yielded absorption maxima at 450 and 470 nm.

Two solvent systems were used to develop the chromatograms, but color was lost as the chromatograms developed and the solutions eluted from bands on the chromatograms were light yellow or colorless. From these eluted bands, she found absorption only below 350 nm. For chromatography, two solvent systems were employed: a) BAW (butanol/acetic acid/water) 6:1:2 (Seikel, 1962) and b) BAW 3:1:1 (Mabry et al., 1970). As a result of the decolorization observed in chromatography, spot tests of the crude extract were conducted. The extract became colorless with the addition of base and hydroxy quinones therefore were not present. Absorption spectra of the crude extract did not compare with the spectra expected for benzoquinones or naphthaquinones. Sulya believes the pigment is phenolic in nature, perhaps a flavonoid, on the basis of color reactions with acid and base and solubility characteristics.

Besl et al. (1975) have examined the pigments from 30 species of Hygrophorus by chromatography and concluded that the pigments of 18 species are derivatives of muscaflavin (XLIV).

C H A P T E R I I

MATERIALS AND METHODS

Collection of Specimens

The specimens of agarics utilized for the extraction of pigments and related work were collected in Massachusetts, Michigan and Mississippi from 1968 to 1974. Some specimens found in both Ohio and Michigan during 1963-1968 also were studied, but they proved to have limited use due to the deleterious effects of aging and storage. Most collections were made in the period between June and October of each year, but a few were found earlier or later. The number of sporophores in collections was variable as might be expected. At times only single sporophores of a species were found, while at other times dozens occurred.

Upon collection, the specimens were wrapped in waxed paper to keep each collection separate from all others and to prevent dehydration. If it was apparent that pigments could not be extracted within several hours after the specimens were found, the collections were kept cold in a portable ice chest.

After arrival at the laboratory, the collections were carefully examined and any sporophores which showed signs of decomposition were discarded. When possible, one to several sporophores were removed to keep as voucher specimens in the herbarium. These were dried and are on deposit in the Mycological Herbarium at the University of Massachusetts (MASS) in Amherst, MA 01002.

Identification of the collections was made using the monographs of Hygrophorus by Hesler and Smith (1963) and by Orton (1960).

The collections are represented by Cibula collection numbers in the experiments and the detailed data of each collection are found in Appendix B. The species of Hygrophorus collected and studied are listed alphabetically in Appendix B. In addition, certain other agarics were used for comparison of pigments. These also, are listed in this appendix.

Preparation of Crude Extracts

The fresh sporophores, were weighed prior to extraction and then were placed in a large mouthed Erlenmeyer flask. Sufficient methanol or ethanol was added to cover the specimens completely. After 15-60 minutes of steeping, during which time the flask was gently swirled several times, the solution was decanted and filtered into another flask. This process was repeated several times on a collection and all the decanted and filtered solutions were combined. At this point the sporophores were usually colorless. If any color remained (usually some shade of brown), a more lipophilic solvent (petroleum ether, benzene, diethyl ether, acetone or pyridine) was added to determine whether the remaining color was extractable. In specimens where there was still an extractable pigment, this was filtered out but was not combined with the original alcoholic extracts. All extracts were bottled in light-tight containers and capped with air tight stoppers. In most cases, the extracts were immediately processed further as very early in this study it became apparent that

absolutely fresh material was necessary for pigment analysis. As will be shown later, the pigments in Hygrophorus, especially the species closely related to H. miniatus and H. flavescens, were very sensitive to both light and air. Studies on dried materials gave results which were quite different in comparison to studies on fresh material. Additionally, there were differences between recently dried material and material that was several years old or older.

To the methanolic and ethanolic extracts of pigments petroleum ether and sufficient water were added to effect phase separation. Pigments which are of a lipophilic nature tend to be located in the epiphase layer, while those of a more polar nature remain in the hypophasic layer. This treatment of the extracts was done in a separatory funnel with Teflon coated stopcocks because there are difficulties encountered when stopcock grease on ground glass stopcocks is employed. These problems have been documented by Cooney et al. (1966) and by Jameson (1973). With pigments which are quite polar, phase separation will remove other lipophilic contaminants from the pigment which remains in the hypophase layer. Alternatively, the crude extract may be dried in a flash evaporator, as described below, and then the dried residue can be rehydrated with an amount of water sufficient to dissolve all colored compounds. Lipophilic compounds remain as a residue in the flask. Filtration of the rehydrated pigment to remove any suspended material completes the process.

A flash evaporator with a water bath at 35-40^{o}C was used to

concentrate the phase which contained the pigment. After this treatment, it was found advisable to introduce nitrogen into the storage container as the concentrated pigment present in a reduced volume of solvent was found to be much more sensitive to change if allowed to stand under air. It was also found that the extract could be taken to dryness and then stored in darkness in a vacuum jar. This procedure worked very well. For example, a small sample of the purified magenta fraction (rhodohygrocybin)[2] from H. purpurefolius was kept in this fashion for more than a year without any loss of color. Also, several other crude extracts taken to dryness and kept under a vacuum, when later rehydrated and chromatographed, gave results which paralleled those obtained when a fresh preparation of crude extract was used.

There are a number of extractive techniques described in the literature which were not successful with specimens of Hygrophorus. For example, Bertrand (1902) and Gabriel (1965) recommend that the sporophores be placed in boiling ethanol and refluxed for 15 minutes to prevent enzymatic degradation of the pigment. This procedure, when attempted with the Hygrophorus pigments, decolorised a magenta component present in several species and therefore was not used further. Workers with other pigment types (Holden, 1965), have

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[2]At this point, it is well to introduce two pigments which were isolated from a number of Hygrophorii. Neither correlated with any known basidiomycete pigment. One, a magenta colored compound, was named "rhodohygrocybin," while the second, a bright yellow in color, was called "flavohygrocybin." Details concerning these pigments are given later.

experienced similar difficulties, and Robinson (1967) notes that some pigments are destroyed when plant tissues are heated.

Other investigators (Butler, et al., 1965; Holden, 1965; Davies, 1965; Fiasson, 1968) have suggested grinding the plant tissues in a mortar and pestle with washed quartz sand or in a blender. I found that this method was not successful with Hygrophorus species. Several collections of a number of Hygrophorii were immersed in methanol or ethanol and then macerated in a Waring Blender. The extracts darkened rapidly, and when chromatographed, did not separate well into the two major known pigment moities. Instead, there were many other compounds, both colored and colorless which separated on the chromatogram.

Methods of Pigment Separations

Paper Chromatography.

Both Whatman #1 and #3MM papers were used. Although good results were obtained with Whatman #1, the results produced with Whatman #3MM were better because the spots or bands produced were more distinct when seen in a transmission mode. The greater chroma of separated pigments seemed to be a direct result of the greater loading capacity of the 3MM paper.

Several different procedures were followed. For most screening studies and for preparative runs, sheets of Whatman 3MM paper were cut to 57 X 18.5 cm and application of the crude extract was made along a line 8.5 cm from one end of the sheet. When it was desirable

to run a number of different collections simultaneously, the extracts from the specimens were spotted along this line at points separated approximately 2.5 cm from each other and the sides of the sheet. Spot diameter was kept below 1 cm. With freshly prepared extracts, where a sufficient volume of solvent was used to just cover the sporophores in the flask, 20-50 applications over the same spot with drying between applications, generally yielded excellent results. If the crude extract had been concentrated by evaporation such that a solution volume of approximately 5-10 ml resulted from 100 ml of original extract, only one or two applications were necessary. No differences were noted between freshly prepared crude extracts and the more concentrated solutions produced by vacuum evaporation.

When crude extracts were employed to produce band chromatograms for the analysis of a specific pigment moiety, the crude extract was drawn out along the line over the entire width of the paper. Here care was taken so that the width of the band did not exceed 0.5 cm. The prepared sheet was then folded and developed in descending mode in a deep chromatographic developing jar.

For rapid screening purposes, 20 X 20 cm sheets of Whatman #3MM paper were spotted along a line 3-3.5 cm from bhe bottom of the paper. These sheets were then developed in ascending mode in an Eastman Chromatogram developing chamber using the same solvent systems used for the larger, descending development chromatograms.

In evolving a system to separate pigments of Hygrophorus,

H. flavescens, H. miniatus and H. cantharellus were utilized in the early phases of this study. At that time, it was determined that except for those reported by Esborn (personal communication), none of the solvent systems discussed earlier, e.g., Gabriel (1965), were usable. The preliminary screening of several species of Hygrophorus showed that the pigments were freely soluble in water and completely insoluble in acetone. Thus acetone-water mixtures were the most commonly used chromatography solvent systems. Three different acetone-water ratios were used, namely: 1:1, 6:4 and 7:3, all v/v. Early in this study, large volumes of these solvent systems were prepared and then used as needed, but it soon became evident that the R_f values of the pigments were increasing with the age of the solvent systems. The difficulty appeared to be the differences in volatility between the two solvents. Upon standing, the more volatile acetone was partially removed from solution, so that the acetone/water ratio was less than when first prepared. Santavy (1969, p. 423), in fact, notes that solvent mixtures must be prepared just before use. Subsequently only freshly prepared solvent was used for chromatography and no more difficulties were encountered.

In the large development chambers, a dish of acetone was placed in the bottom of the chamber for equilibration purposes. With the Eastman chamber, the solvent was simply poured into the tray when development was to begin. The results using the same solvent system were consistent with each other, but the large sheets were better for analysis due to their greater length and the better resolution

of closely spaced pigments.

For the freely water soluble, non-polyene Hygrophoroid pigments, two other solvent systems were used with some success. One was a 1:1 isopropanol/water mixture (v/v), while the other was an acetone/petroleum ether b.p. 30-60°C water mixture, 50:50:35 v/v/v. Two other solvent systems, 1-butanol/acetic acid/water 4:1:5 v/v/v and ethanol/water, 9:1 v/v were not completely satisfactory as some pigmented material remained at the origin. One other solvent system, 1-butanol/acetic acid/water, 6:1:2 v/v/v was not useful as, on the larger chromatograms, decolorization of the pigments occurred. This aspect was also observed by Sulya (1971).

When band chromatograms were prepared to gain a large quantity of a particular pigment for further analysis, the band of interest was simply cut from the chromatogram and then cut into small pieces. The pigment was then extracted from the strips by water and the solution was filtered to remove suspended fibres. Although Cassidy (1957) suggests the use of a tapered piece of the chromatogram and a subsequent removal of the compound by eluting countercurrently, the above method was more rapid. Extraction proved to be effective as a result of the hydrophilic nature of these pigments and was accomplished easily under nitrogen.

Thin Layer Chromatography (TLC).

Most TLC investigations were made on either the Eastman Silica Gel Type K 301 R2 sheets, or the Gelman ITLC Type SG Chromatography

sheets. In either case, the sheet size was 20 X 20 cm and development was accomplished using an Eastman Chromatogram developing chamber or its equivalent. In one or two isolated instances, laboratory-prepared silica gel plates on glass were also employed.

Eastman Silica Gel sheets gave good separations with the non-polyene, hypophasic hygrophoroid pigments while the Gelman sheets proved advantageous in separating known carotenoids (e.g., canthaxanthin from Cantharellus cinnabarinus) and polyene type pigments. The same solvents which gave good results in the paper chromatographic studies were also used for the Eastman silica gel plates. On the Gelman sheets, to separate the polyene type pigments petroleum ether (b.p. 30-60°C)/acetone mixtures (80:20) v/v, as well as chloroform (Goodwin, 1965) proved to be more satisfactory.

Since most of the compounds of interest were colored they were readily visible under ordinary room lighting conditions. However, observations also were made and recorded using both long wave and short wave ultraviolet illumination in a darkened room. Of the two sources of ultraviolet illumination long wave ultraviolet was much more informative.

Spray reagents were employed to check for functional groups. Ninhydrin (Brenner, et al., 1969; Fahmy, et al., 1961; Patton and Chism, 1951; Moffat and Lytle, 1959) in the form of spray reagent (Sigma NIN-3) was an aid in visualizing ninhydrin sensitive areas on both the paper and TL chromatograms. This reagent is a test for amino acids and some lower peptides. Most amino acids yield violet

colors with this reagent, but proline and hydroxyproline produce yellow colors (Brenner, Niederwieser and Pataki, 1969; Bell and Fowden, 1964).

Extracts from several Hygrophorii were tested for alkaloids. This was done to determine if any of the identifiable pigments were alkaloidal in nature. For this purpose, silica gel plates were spotted and then developed with a mixture of 1-butanol/acetic acid/water 6:1:2 (v/v/v), (Santavy, 1969). Dragendorff's reagent (Munier, 1953; Santavy, 1969) would make alkaloids visible on the TLC plates.

Pauli's reagent (Grimmet and Richards, 1965) was used to examine pigmented areas on the chromatograms for the presence of hydroxy groups.

Additionally, Amido black 10B in methanol/acetic acid 9:1 v/v (Dawson et al., 1969) was applied to selected chromatograms to test for the presence of protein.

For each chromatogram, R_f values were noted for each colored spot and these data were recorded for each species.

Column Chromatography.

Several different column diameters were employed in this study, but the best success was obtained with two: a 1 X 13 cm column and a 1 X 32 cm column. Both were used primarily in isolating rhodohygrocybin and flavohygrocybin from Hygrophorus cuspidatus.

To construct the column, a slurry of Baker TLC Microcrystalline

cellulose (#1529) was prepared with an acetone/water mixture (7:3 v/v) as the solvent. The glass tube of the column was drawn out at one end to form a small diameter exit for the effluent, and this tip was packed with glass wool. The prepared slurry was slowly poured down a glass rod into the tube to promote packing without the formation of air pockets. Immediately after the last of the solution vanished from the top of the packing, a concentrated solution (approximately 5 ml from 100 ml) of the crude extract dissolved in acetone/water, 7:3 was then carefully layered over the packing. After the crude extract was taken into the column, development proceeded using the same mixture of acetone/water used to prepare the slurry. These two columns gave results which were consistent with those obtained with paper chromatography using the 3MM paper. A larger diameter tube (2 cm) was tried later, but the results were not satisfactory. In view of the scanty amount of material available for analysis, and the repeated success with paper chromatography, this method of separating pigments was not pursued further.

Dowex 50, H+ form was used with success to remove ninhydrin sensitive materials from selected extracts. After this resin was prepared in the H+ form (pg. 50), and poured into a column, it was washed with a methanol/water solution 4:1, (v/v) after which the filtered crude methanolic extract was passed through. The eluate was saved, and the column was washed successively with water (approximately 200 ml) and then with 5% ammonium hydroxide in water;

each washing was retained for later processing. Mallinckrodt Amberlite IR-120 H.C.P. resin also was a favorable material for cation exchange column chromatography.

Spectroscopy

Both a Bausch and Lomb "Spectronic 505" and a Cary 17 recording spectrophotometer was used to obtain absorption spectra in the visible and ultraviolet (200-700 nm). The pigments were dissolved in suitable solvents, the choice being determined by the nature of the pigment and the wavelength region being examined. For the extremely polar rhodohygrocybin and flavohygrocybin, only water, methanol and ethanol were suitable. For spectra in the visible regions with some other polar pigments which were isolated, chloroform, pyridine, and diethyl ether were employed in addition to the above solvents. Benzene, petroleum ether, methanol, diethyl ether and chloroform proved to be the best solvents for non-polar carotenoids and with one other non-polar yellow pigment.

Infrared spectra were recorded on a Perkin Elmer 237 B grating infrared spectrophotometer and on a Beckman IR-10 infrared spectrophotometer. The very hygroscopic pigments were mixed with potassium bromide and pelletized. It was found necessary to keep such pigments (e.g., flavohygrocybin and rhodohygrocybin) in a dessicator prior to pelletizing. It was also determined that pelletizing was best accomplished under dry nitrogen. If this procedure were not followed, these very hygroscopic pigments would imbibe sufficient

water to make interpretation of the spectrum quite difficult.

A Varian A 60 spectrometer was used to obtain nuclear magnetic reasonance (NMR) spectra. Since flavohygrocybin is extremely soluble in water and not soluble in the normal organic solvents used for NMR spectroscopy, deuterium oxide (heavy water) had to be the solvent for this pigment. Deuterated chloroform was used for a polyene pigment which exhibited some solubility in chloroform.

Mass spectra were obtained with a Hitachi-Perkin-Elmer RMU 6 L mass spectrometer.

Isolation and Purification of Flavohygrocybin and Rhodohygrocybin

Spraying chromatograms with ninhydrin (Figure 18) revealed the presence of amino compounds which interfered with the separation of both flavohygrocybin and rhodohygrocybin. These amino compounds were present in the bands of rhodohygrocybin and flavohygrocybin which had been separated from the crude extract only by preparative paper chromatography. It was found that these amino compounds could be removed from the extract by treating a methanol/water (7:3 or 8:2 v/v) solution of the crude extract with a strongly acidic cation ion exchange resin column, in hydrogen form (Dowex 50, H+ or Amberlite IR-120 H.C.P.).

As an example, a typical run employing an extract of H. miniatus f. longipes is given. This is shown diagramatically in Figure 2. A collection of fresh sporophores weighing 48.2 g was placed in an

Erlenmeyer flask and approximately 100 ml methanol was added. Nitrogen was allowed to bubble slowly from the bottom of the flask through a narrow diameter glass tube which was introduced into the flask. This entire assembly was enclosed in a glove bag in which the air had previously been replaced with nitrogen. Nitrogen was permitted to slowly bubble through the flask for one hour, after which time, the methanolic solution was decanted and filtered. This methanolic solution was washed three times with petroleum ether to remove any colorless lipophilic materials. The crystal clear, deep reddish-orange solution which resulted was the crude extract which was processed further.

The Dowex resin used had previously been prepared in the H+ form by washing with 100 ml 2 M hydrochloric acid, followed by 300 ml of deionized distilled water. The resin bed was prepared by pouring the resin in the form of a 4:1 (v/v) water/methanol slurry into a 1 cm diameter glass tube fitted with a sintered glass filter at its base. Sufficient slurry was poured into the tube to form a 5 cm bed. After several more rinses with the methanol/water solvent (4:1 v/v), the column was ready for operation. Alternatively, a water-resin slurry could be poured into the tube followed by a wash with 200 ml of a 4:1 (v/v) methanol/water mixture. As the last of the prewash solvent vanished from the top of the column, the pigment solution was carefully poured in. The first effluent, approximately 5 ml, was colorless, and was discarded. The remaining eluate which passed through the column was collected and was a bright lemon yellow

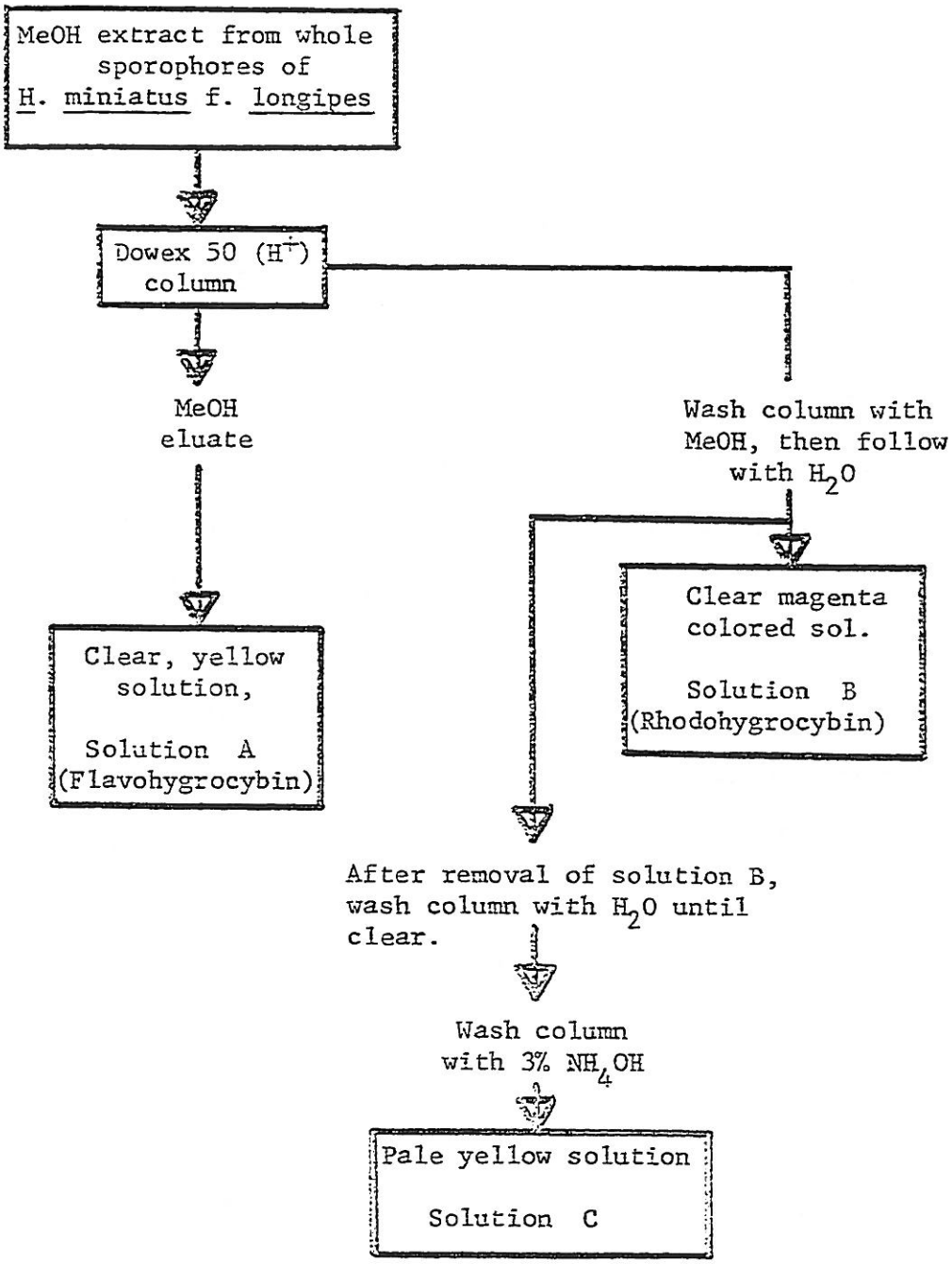

Figure 2. Separation of rhodohygrocybin and flavohygrocybin from other amino compounds present in the crude extract.

in color. This effluent was Solution A in Figure 2. Following this, the column was washed with methanol (approximately 50 ml) until the eluate was clear. At this point, a deep reddish colored band could be observed near the top of the column. The column was then washed with water. At first, the eluate was colorless but it soon became a deep magenta in color. This colored fraction was collected and was Solution B in Figure 2.

The earlier colorless fraction when evaporated to dryness, contained no solids. After removal of the magenta colored compound, further washings were also colorless. The column was washed next with an aqueous solution of ammonium hydroxide (3% NH_4OH) which yielded a pale yellowish solution (Solution C, Figure 2). When tested with ninhydrin this solution was strongly positive and when the solvent was evaporated, colorless needle shaped crystals formed. These were not processed further.

Another technique which also worked well for separating rhodohygrocybin and flavohygrocybin from amino compounds is as follows: In a collection of Hygrophorus miniatus var. miniatus, the fresh sporophores weighed 53.6 g. The crude extract was prepared as previously described except that it was not washed with petroleum ether. Rather, the solvent was removed by means of a flash evaporator at 30°C leaving a dried residue which weighed 0.764 g. This dried residue was rehydrated with 10 ml of distilled, deionized water and then filtered. This yielded a clear, deep, blood-red solution from which the solvent was again evaporated under reduced

pressure at 30°C. The remaining residue was rehydrated with 2 ml of deionized, distilled water and after all the pigment was dissolved, 8 ml of methanol was added. Using a column prepared as above, with the Dowex resin, the blood-red extract was carefully layered on top of the resin bed. After the extract was adsorbed, the column was eluted with methanol/water (8:2 v/v) yielding a lemon yellow colored solution (Solution A, Figure 13). Subsequent treatments were as described above and yielded identical results.

Concentration of Solution A yielded a fraction which was banded on 3MM chromatography paper. Development was with acetone/water as previously described. These chromatograms produced only a single pigmented band, but several ultraviolet fluorescing bands of different R_f values were also present.

Elution of pigment from the excised bands of several runs gave a compound which tested negative when treated with ninhydrin. Subsequent chromatography of the compound using either paper chromatography of the compound using either paper chromatography with the various developing solvents or TLC resulted only in a single spot. When TLC plates were treated to char organic compounds by using sulfuric acid, no additional spots occurred. On this basis, the existence of a reasonably pure single compound at the conclusion of these operations was demonstrated. In the first method described above, the yield of flavohygrocybin was 0.057 g (0.118%) while in the latter, it was 0.065 g (0.121%). Spectral data were obtained on these samples. Concentration of Solution B yielded a deep magenta

colored extract which was also banded on 3MM chromatography paper. A single band resulted when developed. As with flavohygrocybin, excision and subsequent elution of the magenta band produced the compound which was used for spectral data.

Spot Tests for Polyene Pigments

The reactions of the carotenoids in the presence of strong mineral acids to yield blue or violet colored products has been reviewed by Karrer and Jucker (1950). Of particular interest is the reaction of the polyene pigments with concentrated sulfuric acid whereby the acid solution is colored from a greenish-blue in some polyenes through to an intense blue or blue-violet in other polyenes. The addition of water causes this coloration to disappear (Kuhn and Winterstein, 1928). As a consequence, this reaction must be performed in the absence of water. An adaptation of this procedure was developed to test rapidly small amounts of fungus tissue for the presence or absence of polyene pigments.

A small amount of pigmented tissue from the specimen is placed into a depression of a porcelain spot plate and covered with sufficient methanol to nearly fill the depression. The tissue then is macerated with a small glass rod or other suitable instrument and allowed to steep in the methanol for a few minutes. When the methanolic solution has become colored, it is carefully transferred to a clean depression of the test plate. The test is positive for polyenes if, upon addition of concentrated sulfuric acid, an

immediate bluish coloration develops (Kuhn and Winterstein, 1928; Molisch, 1923; Wissilingh, 1915). The actual colors which may be obtained can vary from a deep aqua through shades of blue and purple. The depth of coloration will depend upon the actual amount of pigment in the crude extract while the hue is dependent upon the polyene(s) present. However, it must be recognized that further studies must be performed on the crude extract in order to determine the exact nature of the polyene pigment. It is possible that the absence of the blue coloration does not necessarily mean the absence of polyenes. When crude extracts are being examined there is the distinct possibility that the action of strong mineral acid on some other constituent in the crude extract may result in charring which masks the blue color produced by a polyene. A more accurate determination may be obtained by using purified pigment fractions. The simple use of crude extracts does allow for a rapid screening of agarics, and a positive test is indicative that this pigment extract should be examined in more detail.

The Carr-Price test for polyenes (Carr and Price, 1926) can also be performed in a similar manner. The extraction is accomplished in a spot test plate as described above, but after transfer and evaporation chloroform is added to the dry extract. To this chloroform solution, is added several drops of the Carr-Price reagent (a saturated solution of antimony trichloride in chloroform). As in the above case, a positive reaction is given by the appearance of blue to violet colors in the solution. In practice, this test was performed

at the same time as the sulfuric acid test. For this it is necessary to use three spot depressions: One to prepare the crude extract, one for testing the extract by sulfuric acid and one for testing the extract by the Carr-Price reagent.

Photography

Photographs were taken of the fungi as they occur in the field and of their pigments on the chromatograms. A single lens reflex Exacta VXIIa camera equipped with an f/2.0 58 mm Carl Zeiss, Jena "Biotar" lens was used.

In all cases, the film used was either Kodak Ektacolor CPS or Kodak Kodacolor, both 35 mm films. Both are color negative films and these were processed with minimum equipment using Eastman C-22 chemistry (Eastman Kodak, 1965 and 1971). Negative color films were used in order to obtain more accurate color balance than can be obtained from several other films (e.g. Kodachrome X or Ektachrome) under the same types of illumination. Negative color film also had the advantage of convenient processing into either transparencies or prints.

In the field, either sunlight or portable electronic flash was used to expose the negatives. An HCE Vari-Close-Up lens was used with the normal camera lens to permit photography at near unity magnifications.

To record the daylight aspect of the chromatographed pigments, the chromatograms were illuminated from behind, and the photograph

taken of the chromatogram in a transmission mode. Here, to facilitate later darkroom work, an 80B filter was used over the taking lens since this filter converts tungsten illumination of an approximate color temperature of 3400° K to that of daylight (5500° K).

To photograph the fluorescent aspect of the separated pigments, the chromatogram was illuminated with long wave ultraviolet illumination produced from a Blak-Ray Model B100 lamp. The taking lens was filtered with a Kodak HF-2 ultraviolet absorbing filter in order that only the visible light fluorescence would photograph. If this filter is not used the ultraviolet illumination would record as a high intensity blue light. Even with this arrangement, filter changes in the enlarger were necessary to print the photographs. Color compensating filters were used and the prints were made on Ektacolor RC paper processed by Kodak Ektaprint Chemistry (Eastman Kodak Company, 1970).

C H A P T E R I I I

RESULTS OF EXPERIMENTS

Species of Hygrophorus

The results of chromatography of the crude extracts of the various agarics studied are detailed below. Color terminology given for the bands separated as seen under visible light is that used in the ISCC-NBS system of nomenclature (Kelly and Judd, 1955; Kelly, 1965; Inter-Society Color Council-National Bureau of Standards, 1965). The only exception is the name "magenta" which is a well established term in the description of color photographic processes. This term corresponds to purplish-red in the ISCC-NBS nomenclature.

The intensities of the bands on the chromatograms are given as follows: VW = very weak; W = weak; M = medium intensity; S = strong; VS = very strong (or very intense). The data for the collections studied are given in Appendix B. In the column denoting the R_f value, rhodohygrocybin is designated by a single asterisk (*), while flavohygrocybin is designated by a double asterisk (**).

Hygrophorus flavescens (Kauffman) Smith and Hesler. Lloydia 5:60. 1942.

Figures 3, 14, 15, 17, and 18.
Tables 1, 2, 3, and 38.

Hesler and Smith (1963) observed that this species varies from pale sulphur yellow to orange in color of the pileus but is never red. I also have found forms which were typically orange, fading to yellow

in age, as well as specimens which were entirely yellow without ever a hint of orange. Collections which had orange pilei were much more common than those of the yellow form. Chromatography of absolutely fresh extracts from the orange forms gave the separations noted in Tables 1 and 2, while the data from yellow forms are presented in Table 3. The latter show almost a complete absence of the magenta band. Fluorescence of the separated pigments under long wave ultraviolet is evident in Figure 15.

Hygrophorus cantharellus (Schweinitz) Fries. Epicrisis Systematis Mycologici p. 329. 1838.

Table 4, and 38.

Historically, this species has been thought to be close to the genus Cantharellus, and the distant, arcuate decurrent lamellae as well as the colors do present rather a cantharelloid aspect. Fries (1838) places H. cantharellus with C. cinnabarinus (which he considered to be a Hygrocybe!) and notes a similarity in color to that of C. cibarius.

Pigment separations with this species are quite close to those observed in the orange form of Hygrophorus flavescens except that the amount of rhodohygrocybin present is greater. This pigment thus appears to be responsible for redder coloration of the pileus and stipe of H. cantharellus.

Hygrophorus miniatus (Fries) Fries var. miniatus. Epicrisis Systematis Mycologici p. 330. 1838.

Tables 5, 6, and 38.

Figure 3. Hygrophorus flavescens, orange form. X1.

In the tables which follow, abbreviations where necessary, were used. Their equivalents are given below:

p. = pale	v.p. = very pale	brill. = brilliant
l. = light	or. = orange	mod. = moderate
gr. = green or greenish	bl. = bluish	oy. = orange-yellow

TABLE I

HYGROPHORUS FLAVESCENS, ORANGE FORMS

ACETONE/H_2O 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0			p. whitish	W
2	0.09	p. yellow	VW	p. bluish-white	W
3	0.10*	magenta (light purplish-pink)	M	rose to reddish-orange	M
4	0.11	p. yellow	W	yellow	W
5	0.20			p. yellow	VW
6	0.40**	vivid yellow flavohygrocybin)	VS	chartreuse (strong yellow-green)	S
7	0.50			blue	W

TABLE 2

HYGROPHORUS FLAVESCENS, ORANGE FORMS

ACETONE/H_2O 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.22	p. yellowish-orange	W	p. yellow	W
2	0.23*	magenta	M	rose to yellowish-orange	M
3	0.25	pale yellow	W	v.p. yellow	VW
4	0.34	pale yellow	W	p. yellow	W
5	0.41	pale yellow	W	v.p. yellow	VW
6	0.50**	vivid yellow	VS	chartruese (strong yellow-green)	VS
7	0.57			bluish	W

TABLE 3

HYGROPHORUS FLAVESCENS, YELLOW FORMS

ACETONE/WATER 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0			pale greenish blue	W
2	0.04	v.p. orange	VW	p. orange yellow	W
3	0.07*	v.p. magenta	VW	p. rose to p. orange yellow	W
4	0.11	yellowish-white	VW		
5	0.21	grey	W	dark	
6	0.36	p. yellow	W	p. yellow green	W
7	0.45**	vivid yellow	VS	chartreuse	VS

TABLE 4

HYGROPHORUS CANTHARELLUS

ACETONE/WATER 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.10*	magenta	S	rose	M
2	0.12	yellow	M	yellow green	M
3	0.20	v.p. yellow	VW	gr. yellow	VW
4	0.46**	vivid yellow	VS	bright chartreuse	S

This agaric, in contrast to H. flavescens, often has a brilliant scarlet pileus and stipe, but fades through orange to yellow as it ages in the same manner as the orange H. flavescens. Collections of scarlet sporophores yielded the pigment separations presented in Tables 5 and 6. Separations are similar when an isopropanol-water mixture is used as the developing solvent instead of acetone.

The amount of rhodohygrocybin is variable according to the age of the sporophore, while flavohygrocybin remains quite constant. If one collects young, vigorously developing sporophores which are colored a rich scarlet to red, rhodohygrocybin is found in highest concentration. From these sporophores there is a minimal amount of the pigments on either side of the magenta band (R_f 0.25 and 0.28, acetone/water 6:4). In older collections these bands become intensified with a concomitant reduction of rhodohygrocybin. There is little diminution of flavohygrocybin (R_f 0.50, acetone/water 6:4).

Spraying the chromatograms with Pauli's reagent gave positive reactions at the site of all pigmented areas, indicating perhaps that all of these pigments are hydroxylated. Spraying the chromatogram with a ninhydrin spray showed large areas of ninhydrin sensitive material which did not necessarily correlate with the pigmented spots (Figure 18). Likewise, there did not appear to be much correlation between the spots which were positive with Pauli's reagent and those that were ninhydrin positive. The ninhydrin sensitive areas indicate that there are amino compounds which also separate and appear in the chromatograms.

TABLE 5

HYGROPHORUS MINIATUS var. MINIATUS

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.25	p. yellow	W	yellowish	W
2	0.26*	magenta	S	strong pink	M
3	0.28	light yellow	M	yellowish-green	W
4	0.31	p. yellow	W	greenish-blue	W
5	0.39	p. yellow	W	greenish-blue	W
6	0.50**	vivid yellow	S	bright chartreuse	S

TABLE 6

HYGROPHORUS MINIATUS var. MINIATUS

ISOPROPANOL/WATER 1:1 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.38	v.p. yellow	VW	yellowish	VW
2	0.40*	magenta	S	rose (strong pink)	M
3	0.43	light yellow	M	p. yellowish-green	W
4	0.52	p. orange yellow	W		
5	0.60**	vivid yellow	S	yellow-green	S
6	0.67	p. yellow	W	p. greenish-blue	W

Several chromatograms were prepared and developed using n-butanol/acetic acid/water, 6:1:2 (BAW 612); this solvent was used as it has been shown to be successful in separating a variety of plant alkaloids (Santavy, 1969). With this solvent the pigmented areas were destroyed when long developing times were employed. With shorter development times, followed by spraying with Dragendorff's reagent, no positive areas indicating alkaloids at the sites of the pigmented spots on the chromatograms were observed. There was a bright spot at the origin, a major spot located between the spot of rhodohygrocybin and the spot representing flavohygrocybin, and also two strongly positive spots located below the flavohygrocybin. (The order of pigment separation with BAW 612 is the same as above.)

Hygrophorus miniatus f. longipes Smith and Hesler. Sydowia 8:321. 1954.

Figures 17 and 18.
Tables 7, 8, and 38.

With this species also, the crude extract in acetone/water separates into two major components, viz rhodohygrocybin and flavohygrocybin. When isopropanol was used as a solvent, again these two pigments separated.

In addition to rhodohygrocybin and flavohygrocybin, other pigments in much smaller amounts were also observed to separate. These are evident in Table 7. Since these pigments are present in only very small amounts, their detectability depends on the amount of extract used to spot the origin. If only a small amount of crude

TABLE 7

HYGROPHORUS MINIATUS f. LONGIPES

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.16	p. orange	W	p. orange	W
2	0.18	mod. orange	M	gr. yellow	W
3	0.19	mod. orange	M	greenish	W
4	0.20*	magenta	S	rose	S
5	0.21	brill. yellow	M	greenish	M
6	0.26	brill. yellow	M	yellow-green	M
7	0.32	brill. yellow	M	p. yellow	W
8	0.41	p. yellow	W	v.p. yellow	VW
9	0.48**	vivid yellow	VS	bright chartreuse (strong yellow-green)	VS

TABLE 8

HYGROPHORUS MINIATUS f. LONGIPES

ISOPROPANOL/WATER 1:1 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.32	v.p. yellow	VW	p. bluish	VW
2	0.42*	magenta	S	orange-rose	S
3	0.63**	vivid yellow	VS	brilliant chartreuse (strong yellow-green)	VS

extract is used, only rhodohygrocybin and flavohygrocybin will be seen, but if repeated applications are made before development, then these other weakly pigmented spots become evident.

Hygrophorus cuspidatus Peck. Torrey Bot. Club Bull. 24:141. 1897.

Table 38.

A collection of this agaric from Massachusetts gave separations which were identical to those observed for H. miniatus (flavohygrocybin, R_f = 0.49; rhodohygrocybin, R_f = 0.25, acetone/water, 6:4, 3 MM paper). In addition to the usual procedure of paper chromatography, a portion of the extract was run through a column containing Baker TLC Microcrystalline Cellulose as described earlier. The separations of rhodohygrocybin and flavohygrocybin were of the same order as observed using 3 MM paper.

Hygrophorus coccineus (Fries) Fries. Epicrisis Systematis Mycologici. p. 330. 1838.

Figures 14-3, 15-3.
Tables 9 and 38.

This species differs from H. puniceus in not possessing a gelatinous pellicle and from both H. miniatus var. miniatus and H. strangulatus in not having a scurfy pileus. With respect to pigment content and separation, the separations and intensities observed for H. strangulatus are directly comparable as shown in Figures 14-3 and 15-3. The observed separations are shown in Table 9.

Hygrophorus puniceus (Fries) Fries. Epicrisis Systematis Mycologici. p. 331. 1838.

Figures 4, 5, 14-1, and 15-1.
Tables 10 and 38.

The rather large size, viscid pileus, deep blood-red color and the somewhat fibrillose striate margin are the important diagnostic characters which separate this species from the others which have been considered thus far. Here again, there is a repetition of the pattern of pigment distribution detected in the species previously described. In addition, the separations from an acetone/water mixture were nearly identical to that observed for both H. strangulatus and H. coccineus as shown in Figures 14 and 15. This is of interest since these differ from H. puniceus by not having a gelatinous pellicle.

An interesting specimen of H. puniceus was found on 5 October, 1969. The single sporophore was found in a mixed deciduous woodland, and one-half of the pileus was covered tightly with a dense matting of leaves. This side of the cap was found to be non-pigmented (Figure 4). Also, the lamellae beneath the shaded portion of the pileus were not mature (Figure 5). In contrast, the lighted side of the pileus was red and the lamellae beneath were well developed and shed mature basidiospores.

This raises the distinct possibility of light-induced pigment production through the absorption of shorter wavelength radiation by the precursor of the red coloration. There is also a possibility that such a light-induced mechanism may affect the developmental morphology of the pileus. To examine these hypotheses in more detail,

TABLE 9

HYGROPHORUS COCCINEUS

ACETONE/WATER 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.13	l. orange	W	orange	-
2	0.15*	magenta	M	rose to orange-yellow	-
3	0.16	pale yellow	W	p. bluish	-
4	0.18	pale yellow	W	p. chartreuse	-
5	0.23	yellow-grey	W	dark band	-
6	0.45**	vivid yellow	VS	strong yellow-green	-

TABLE 10

HYGROPHORUS PUNICEUS

ISOPROPANOL/WATER 1:1 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.40	p. yellow	W	yellowish	W
2	0.43*	magenta	S	rose	S
3	0.45	l. orange-yellow	M	green-yellow	M
4	0.55	dark	-	dark	-
5	0.63**	vivid yellow	VS	chartreuse (strong yellow-green)	S

Figure 4. Hygrophorus puniceus, X2, collected in a deciduous woodland, South Deerfield, Massachusetts, 5 October, 1969.

The side of the pileus shown here had been covered with a heavy matting of leaves. This area had not developed the pigments responsible for the bright red color which was found on the side of the pileus which was not covered with leaves.

Figure 5. Hygrophorus puniceus, X3, showing the lamellae of the same sporophore shown in the preceeding figure. The well developed lamellae on the right were beneath the pigmented uncovered portion of the pileus. These lamellae produced mature basidiospores. The lamellae on the left side were beneath the covered portion of the pileus, and were still juvenile. No mature basidia or basidiospores were observed on these lamellae.

the pileus was split into two fractions for pigment analysis. The red pigmented portion showed the same pigment breakdown presented in Table 10, but the non-pigmented side lacked rhodohygrocybin and showed only trace amounts of flavohygrocybin. However, in an acetone/water 7:3 developing solvent system, there were two ultraviolet fluorescent compounds which were not present from the extract of the pigmented portion of the pileus. The first, just above the trace amount of flavohygrocybin (R_f = 0.43) had a R_f value of 0.54. The second, an intense bright blue fluorescent spot, was R_f = 0.73. It is possible that these colorless ultraviolet fluorescent compounds may be precursors of the yellow and magenta pigments found in mature sporophores. With this collection I observed, as had Sulya (1971) with H. marchii, a white precipitate which settled out after the crude extract had stood overnight. This precipitate has been observed with other extracts of Hygrophorus but to a much lesser degree, and it does not appear to be related to the pigments found in these agarics.

Hygrophorus parvulus Peck. New York State Museum Ann. Rept. 28:50. 1879.

Tables 11 and 38.

The outstanding feature of this agaric as first documented by Peck (1907) is the reddish color of the stipe in contrast to the yellow pileus. These appear to be constant characters. To study whether or not this reddish coloration in the stipe was caused by the presence of rhodohygrocybin, while the pileus had only yellow

TABLE 11

HYGROPHORUS PARVULUS

ACETONE/WATER 7:3

PILEUS

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.16	or. yellow	W	yellow	W
2	0.56**	bright yellow	M	chartreuse	M

STIPES

1	0.14*	magenta	M	rose	M
2	0.16	or. yellow	M	yellow	W
3	0.56**	bright yellow	M	chartreuse	M

pigments, the sporophores were separated into stipes and pilei and then processed separately.

With acetone/water 7:3 as the developing solvent, the extract from the stipes separated rhodohygrocybin at R_f = 0.14 and flavohygrocybin at R_f = 0.56. The extract from the pilei yielded flavohygrocybin at R_f = 0.56 but no rhodohygrocybin. Both extracts showed a weak orange-yellow spot at R_f = 0.16.

Therefore, the reddish coloration of the stipe results from the presence of both rhodohygrocybin and flavohygrocybin while in the yellow pilei, rhodohygrocybin is absent.

Hygrophorus conicus (Fries) Fries. Epicrisis Systematis Mycologici. p. 331. 1838.

Tables 12, 13, and 38.

Hesler and Smith (1963) describe this species as having a considerable variation in color. Two of the color forms were studied and the results are shown in Tables 12 and 13. One was yellowish-orange in the pileus with an overall olivaceous cast, the other, reddish-orange in the pileus. With both, the methanolic solution after extraction was dark colored - to such an extent that there was little hint of any pigment present. The sporophores after extraction were black.

It is obvious that the pigment separations shown in Tables 12 and 13 correlate with the other species observed thus far. The specimens which have yellowish-orange caps were characterized by lesser amounts, or a total absence, of rhodohygrocybin. More

TABLE 12

HYGROPHORUS CONICUS, ORANGE FORMS

ACETONE/WATER 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0	dark grey	S	dark	-
2	0.04	p. orange	W	yellow	W
3	0.07	light orange	M	yellow-orange	M
4	0.22	v.p. yellow	VW	v.p. gr. yellow	VW
5	0.46***	vivid yellow	VS	brilliant chartreuse	VS
6	0.91	grey	S	dark	-

TABLE 13

HYGROPHORUS CONICUS, RED FORMS

ACETONE/WATER 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long UV	
1	0	dark grey	S	dark	-
2	0.04	med. orange	M	yellow	M
3	0.10*	magenta	M	v.p. rose	W
4	0.12	v.p. yellow	VW	gr. yellow	VW
5	0.26	p. yellow	W	p. yellow	W
6	0.46**	vivid yellow	VS	brilliant chartreuse (strong yellow-green)	VS
7	0.94	grey	M	dark	

importantly however, is the fact that the blackening when handled or bruised does not appear to relate to changes in the group of pigments under study. Instead, the blackening appears to be due to oxidative changes in a previously colorless compound(s). This is evident in the chromatograms where the pigments were recovered undiminished in intensity while some of the other oxidized compounds present in the crude extract were found at the origin and R_f = 0.91-0.94. This hypothesis is further substantiated by observations in the field for sporophores of H. conicus which have stipes that are white at the base also blacken readily in this non-pigmented area when handled or bruised.

Hygrophorus acutoconicus (Clements) Smith var. acutoconicus. North American Species of Mycena, p. 472. 1947.

Tables 14 and 38.

This species differs from H. conicus by not blackening when bruised, by having only yellow to orange-yellow colors and by having a definite, viscid pileus which does not tend to become dry and glabrous as does H. conicus. (H. conicus has a poorly organized gelatinous cuticle 20-35μ thick while there is a well developed gelatinous cuticle 30-50μ thick present in H. acutoconicus var. acutoconicus.) The results of pigment separations are given in Table 14.

H. acutoconicus had the strong band indicative of flavohygrocybin (chartreuse UV fluorescence, λ_{max} 420 nm) found in the other species

TABLE 14

H. ACUTOCONICUS var. ACUTONICUS

ACETONE/H_2O 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.07	p. yellow-orange	W	v.p. yellow	VW
2	0.12*	v.p. orange	VW	v.p. orange	VW
3	0.13	light yellow	M	yellow	M
4	0.25	light yellow	MW	gr. yellow	W
5	0.42**	vivid yellow	VS	brilliant chartreuse (strong yellow-green)	S

TABLE 15

HYGROPHORUS PURPUREOFOLIUS

ACETONE/H_2O 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.15*	magenta	VS	rose	VS
2	0.16	l. orange-yellow	M	yellow	M
3	0.26	p. yellow	W	yellow	W
4	0.46	p. yellow	W	green-yellow	W
5	0.54**	vivid yellow	S	brilliant chartreuse (strong yellow-green)	S

studied previously. However, further interpretations of the chromatogram may not be reliable since the extract was not used promptly. As has been already observed, rhodohygrocybin (R_f = 0.10-0.12 acetone/water 7:3), is quite unstable and upon its destruction the orange band (R_f = 0.11-0.14, acetone/water 7:3) appears to strengthen. Since this was the only collection obtained during the study period, it is included as it definitely has flavohygrocybin and thus has some relationship to the other species studied.

Hygrophorus purpureofolius Bigelow. Rhodora 62:190. 1960.

Figure 6.
Tables 15 and 38.

Bigelow in Bigelow and Barr (1960) observed that the purplish color of the gills is unusual for a species in Hygrocybe. Since as it already has been determined that there are two primary chromophoric moieties which are responsible for the vivid colors in species of Hygrocybe, it seemed pertinent to study the pigments in this agaric as well. One of these compounds, rhodohygrocybin, is somewhat purplish (magenta) in color, and it might be present in H. purpureofolius in greater abundance than in the other species considered thus far. H. purpureofolius was collected only once, but it was found in considerable abundance. The pigment separations from freshly prepared crude extracts are shown in Table 15, and indeed rhodohygrocybin proved to be more abundant in this Hygrocybe than in any others sampled.

Figure 6. Hygrophorus purpureofolius, X1. The purplish gills ("pale vinaceous drab," at times nearly "deep dull lavender," Ridgway, = 2.5R 6.5/1.8 and 6P 5.9/3.7 Munsell) are unusual for species in Hygrocybe. Pigment analysis demonstrates that rhodohygrocybin is indeed, much more abundant in this Hygrocybe than in any others considered in this study.

Hygrophorus strangulatus Orton. New check list of British agarics and boleti. III. Trans. Brit. Mycol. Soc. 43:266. 1960.

Figures 7, 14-2, and 15-2.
Tables 16 and 38.

This brilliantly colored agaric differs from H. miniatus var. miniatus by having constricted spores and by frequently producing clumps of basidiocarps.

Separation of the crude pigment extract from this agaric shows that the brilliant coloration is due to the presence of two major pigmented compounds. Flavohygrocybin, a vivid yellow pigment, is rather persistent and can be found in the sporophores at almost all stages of development. Rhodohygrocybin, a rich magenta (ISCC-NBS: Strong purple red) pigment, is much more unstable even in the sporophore, and tends to vanish with age, causing the more orange and yellow colors often noted in over mature basidiocarps.

Also worthy of note is a colorless, strong blue-fluorescing compound, R_f - 0.54, which was particularly evident from collection #201. This contained a group of brilliantly and deeply colored sporophores which were rather immature. This band was never detected in old specimens of any species, but has been found occasionally in almost all the species here considered. When found, the fluorescing compound was always extracted from young, vigorously developing sporophores. This colorless compound also was found most abundantly in the portion of the pileus of Hygrophorus puniceus which was covered by leaves and had virtually no pigment development (Figure 4). One might speculate whether or not this compound is a

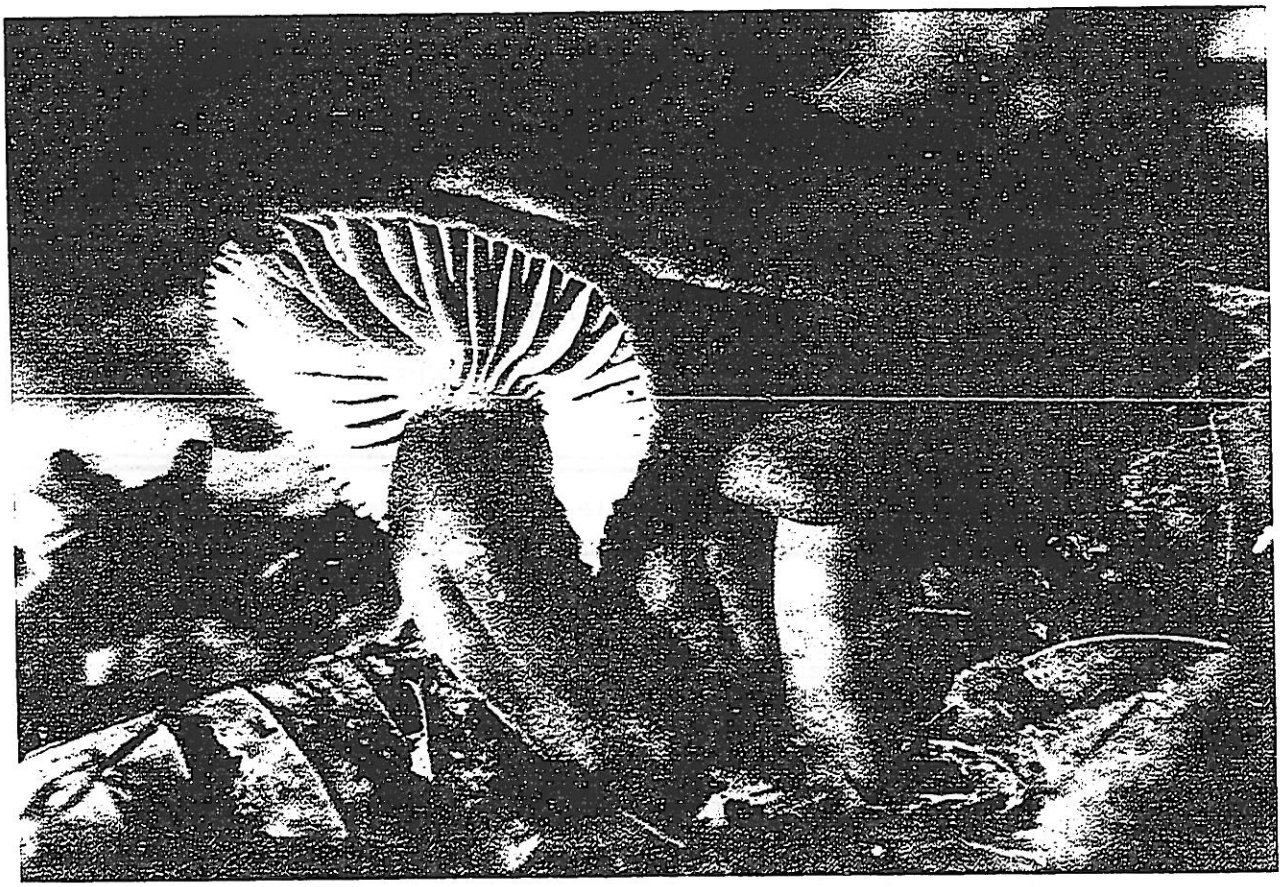

Figure 7. Hygrophorus strangulatus, X2. Separation of the crude pigment extract from this agaric shows that the brilliant coloration is due to the presence of two major pigmented compounds. Flavohygrocybin, a vivid yellow pigment is rather persistent and can be found in the sporophores at almost all stages of development. Rhodohygrocybin, a rich magenta (ISCC-NBS: Strong purple red) pigment, is much more unstable even in the sporophore, and tends to vanish with age, causing the more orange and yellow colors often noted in over mature basidiocarps.

Also, note the more yellow coloration of the immature sporophore on the right. Young sporophores such as this when studied separately, show reduced amounts of rhodohygrocybin, large amounts of flavohygrocybin plus a colorless, strong blue fluorescing compound which is never found in mature basidiocarps.

TABLE 16

HYGROPHORUS STRANGULATUS

ACETONE/H_2O 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.04	p. orange	W	or. yellow	W
2	0.10*	magenta	VS	rose	S
3	0.13	p. yellow	W	yellow	VM
4	0.23	yellowish-grey	M	dark	-
5	0.35	p. yellow	VW	p. yellow-green	W
6	0.42**	vivid yellow	S	brilliant chartreuse (strong yellow-green)	S
7	0.54	--		blue	M

precursor of flavohygrocybin.

Hygrophorus turundus var. sphagnophilus (Peck) Hesler and Smith. North Am. Species of Hygrophorus. p. 145. 1963.

Tables 17 and 38.

An opportunity to examine this agaric occurred when the alkaloids of various Hygrophori were being investigated. As a result, BAW 612 was used as the developing solvent rather than the more usual acetone/water mixture. With BAW long development times always led to complete pigment decolorization as mentioned previously. However, with short development times separations could be achieved before appreciable decolorization took place. The separation for H. turundus var. sphagnophilus is shown in Table 17 and the results are in the same order as observed for other species when the acetone/water developer was used. When H. miniatus f. longipes and H. miniatus var. miniatus were run on the same chromatogram, there were similar results except that in the latter, the R_f for rhodohygrocybin was slightly higher. This difference might be a result of spotting the extract from H. turundus var. sphagnophilus too near the edge of the plate. The chromatograms of H. miniatus var. miniatus and f. longipes would be expected to be similar, since f. longipes is considered to be a form of H. miniatus. This Hygrophorus has been considered by several investigators to be closely related to H. miniatus (Peck, 1901; Hongo, 1952).

TABLE 17

HYGROPHORUS TURUNDUS var. SPHAGNOPHILUS

BAW 612 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0	light orange	M	orange	M
2	0.02	brill. yellow	MS	yellow	M
3	0.04*	magenta	S	rose	S
4	0.10	brill. yellow	S	yellow	S
5	0.19	p. yellow	W	p. yellow	W
6	0.20	p. yellow	W	p. yellow	W
7	0.21	p. yellow	W	p. yellowish-green	W
8	0.24**	vivid yellow	VS	brill. chartreuse	VS
9	0.28	yellowish white	VW	bluish	M

Hygrophorus sp., Cibula # 485.

Tables 18 and 38.

This Hygrophorus from Mississippi appears to be closely related to H. firmus var. trinitensis (Dennis, 1953; Hesler and Smith, 1963) but differs from this species in the color of the lamellae. Collection data and a complete description are in Appendix C.

The observed pigment separations are shown in Table 18. Although only two small sporophores were used to prepare this extract, the intense magenta band is striking. Previous to this collection, large amounts of rhodohygrocybin have been observed only in H. purpureofolius. Also notable is the rather strong yellow band, $R_f = 0.24$. This band is more intense in this collection than has been observed in other Hygrophori which possess flavohygrocybin.

The orange and yellow bands which often are found on either side of rhodohygrocybin were not observed in the developed chromatogram of this collection. Since only a very small amount of material was available for pigment analysis, it is quite possible that if the paper could have been more heavily spotted, these bands as well as others which have been observed in other collections would have been detectable.

Hygrophorus sp., Cibula # 489.

Tables 19 and 38.

This Hygrophorus, which is probably close to H. appalachianensis Hesler and Smith, differs from this and the Hygrophori studied by

TABLE 18

HYGROPHORUS sp., # 485

ACETONE/WATER 6:4

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.18*	magenta	VS	rose	S
2	0.24	brill. yellow	M	p. yellow	W
3	0.48**	vivid yellow	VS	chartreuse	S

TABLE 19

HYGROPHORUS sp., # 489

ACETONE/H_2O 6:4

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.15*	magenta	S	rose	S
2	0.20	p. yellow	W	whitish	M
3	0.33	--		dark band	
4	0.55**	vivid yellow	VS	chartreuse	VS

having a two layered cutis. The uppermost layer having a total thickness of $\simeq 75\mu$ is a trichodermium of nearly hyaline cells. Beneath this is found a layer of strongly pigmented cells with a radial orientation. Rhodohygrocybin and flavohygrocybin are demonstrated to be present as shown in Table 19. A complete description is found in Appendix C.

Hygrophorus subminutulus (Murrill) Orton. Trans. Brit. Mycol. Soc. 43:186. 1960.

Tables 20 and 38.

Sporophores of H. subminutulus are characterized by having both a viscid pileus and a viscid stipe, brilliant red coloration which is persistent, small spores and a small size (Orton, 1960; Hesler and Smith, 1963). The occurrence of this species late in the season (9, December) on the Gulf Coast agrees with Murrill's collection from Florida (1940). The pigment separations shown in Table 20 demonstrate that this agaric has an affinity with H. miniatus.

Hygrophorus speciosus Peck var. speciosus. N. Y. State Mus. Ann. Rept. 29:43. 1878.

Tables 21 and 38.

This brightly colored mushroom, mycorrhizal with larch, invited study even though investigators have not considered it to belong to Hygrocybe. Hesler and Smith (1963) place H. speciosus in subsection Hygrophorus of section Hygrophorus by virtue of the divergent hymenophoral trama and the viscid stipe. The pigments extracted

TABLE 20

HYGROPHORUS SUBMINUTULUS

ACETONE/H_2O 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.12	orange	MW	salmon	M
2	0.16*	deep magenta	VS	deep rose	VS
3	0.19	p. yellow	W	orange-yellow	M
4	0.24	p. yellow	W	v.p. chartreuse	W
5	0.31	mod. yellow	M	dark	-
6	0.53**	vivid yellow	VS	brill. chartreuse	VS

well with MeOH, but were not soluble in nonpolar organic solvents. In this respect, the pigments parallel the behavior of those found in the other species above. With acetone/water as the developing solvent, paper chromatography yielded the results given in Table 21.

Although the separations were very good there were no bands which corresponded exactly with any of the other pigments extracted. Some resemblances are seen, particularly in the chartreuse fluorescence of several of the bands; the pigment of $R_f = 0.42$ appears to be close to flavohygrocybin although the R_f is somewhat low for this solvent system. Elution of this band and subsequent spectrophotometry showed a pigment which had a broad absorption band with a λ_{max} of 488 nm. This absorption was not the same as that determined for flavohygrocybin.

Hygrophorus hypothejus (Fries) Fries. Epicrisis Systematis Mycologici. p. 324. 1838.

Tables 22 and 38.

Hesler and Smith (1963) have observed that at times it is impossible to distinguish species of section Hygrophorus from those of section Hygrocybe on the basis of macroscopic characters only. Microscopically, the bilateral gill trama found in species of section Hygrophorus separates them from Hygrocybe which has species with parallel gill trama. These authors believe that there is a true phylogenetic relationship between these sections and that Hygrophorus is the more advanced.

TABLE 21

HYGROPHORUS SPECIOSUS var. SPECIOSUS

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.07	v.p. yellow	VW	p. chartreuse	VW
2	0.30	orange	VS	or. yellow	S
3	0.32	chrome yellow	S	chartreuse	M
4	0.35	br. yellow	S	dark chartreuse	M
5	0.37	light yellow	M	chartreuse	M
6	0.40	brill. yellow	S	p. aqua	W
7	0.42	vivid yellow	VS	chartreuse	VS
8	0.49	light yellow	M	bright aqua	S
9	0.56	light yellow	M	rust yellow	M
10	0.62	light yellow	M	p. chartreuse	W
11	0.70	p. yellow	W	bluish-white	W
12	0.89	---		bluish-white	M

TABLE 22

HYGROPHORUS HYPOTHEJUS

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.08	----		blue	M
2	0.13	p. yellow	M	yellow	M
3	0.18	----		bluish-white	S
4	0.20	brill. yellow	S	yellow	S
5	0.32	----		bluish-white	S
6	0.48**	p. yellow	W	p. chartreuse	W
7	0.77	----		bluish-white	S
8	0.98	----		bluish-white	S
9	1.00	brown	M	brown	M

Hygrophorus hypothejus has dull cinereous colors but can exhibit yellowish to reddish colors, particularly in age. In order to determine if these brighter pigments have any relationship to the pigments found in other species of section Hygrocybe, extracts were chromatogrammed and the results are in Table 22. Immediately striking are the large number of colorless but strongly fluorescing bands. In addition, the weak yellow colored pigment present at R_f = 0.48 which fluoresces chartreuse under long wave ultraviolet illumination suggests that flavohygrocybin may be present in only small amounts.

Hygrophorus marginatus var. concolor Smith. Papers, Mich. Acad. Sci., Arts and Letters. 38:59. 1953.

Hygrophorus marginatus Peck var. marginatus. N. Y. State Mus. Ann. Rept. 28:50. 1878.

Tables 23, 35, 37, and 38.

With these two agarics, there is a radical departure in pigment type from the species studied previously. Extraction of the whole mushrooms in methanol or ethanol yielded a yellow colored extract similar in appearance to that observed with H. flavescens, but upon washing this extract with petroleum ether to remove lipid materials the pigment was in the epiphase rather than in the hypophase. Paper chromatograms prepared and developed with acetone/water developing solvents yielded separations (Table 23) quite different from such Hygrophori as H. flavescens. A yellowish-salmon colored spot with some tailing was located near the origin. Under long wave ultraviolet

this spot exhibited practically no fluorescence and only a dull salmon color is observed. A yellow spot was produced near the solvent front. No differences were noted on the chromatograms between var. marginatus and var. concolor.

As this species had pigment separations which were markedly different from the others described, and pigments which were freely soluble in non-polar solvents, the spot test procedure (p. 57) was employed to test for polyenes. This was positive and confirmed that the pigments of Hygrophorus marginatus were markedly different from those found in H. miniatus and its allies.

When the pigment extract was placed on Gelman ITLC Type SG Chromatography sheets and developed with a petroleum ether/acetone solvent (p. 48), there was a distinct yellow band separate from the pale salmon colored band. Rechromatography of the yellow band after elution produced only a single spot by visible and UV illumination as well as by exposure to iodine vapor. Since Hygrophorus marginatus was available only in small quantity and only a small amount of extract could be analyzed, chromatographic purity was the only possible criterion of purity employed in this study. When eluted and analyzed with a spectrometer, the yellow band yielded the typical trimodal curve characteristic of a polyene. These absorption spectra for this pigment in several different solvent systems are shown in Figure 8 and Table 37.

The spectral shifts observed between the absorption spectra in the various solvents are the shifts one would expect to find with

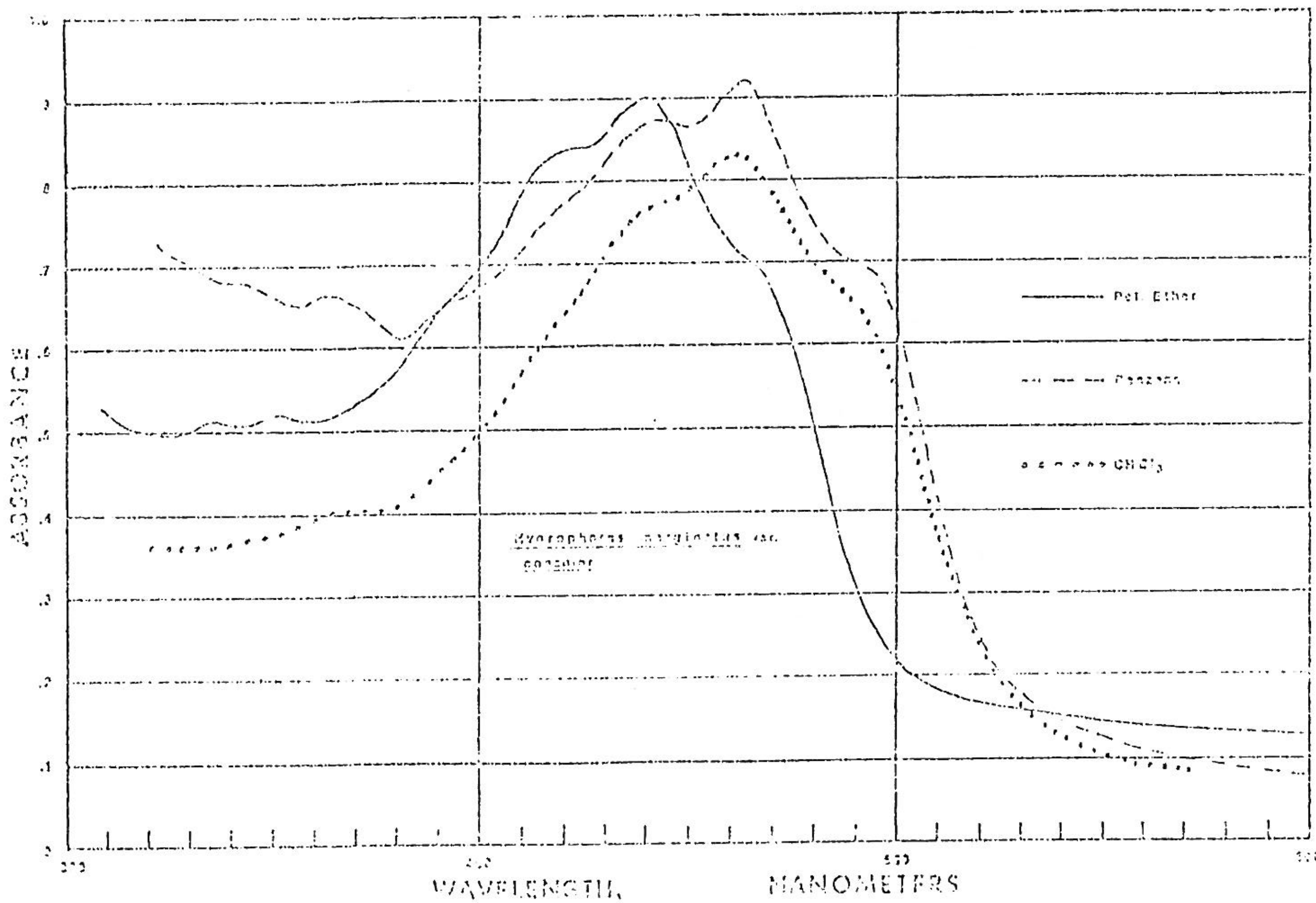

Figure [illegible]. Absorption spectra of the major yellow pigment from *Hygrophorus marginatus* var. *concolor* dissolved in the solvents shown.

polyene-type pigments (Weeden, 1965, 1967, and 1969; Liaaen-Jensen and Jensen, 1965). Spot tests with the pigment as eluted from the chromatograms with both sulfuric acid and antimony trichloride tested positive for polyene pigments.

This pigment is extremely sensitive to alkali and light. The addition of a few drops of a 2% KOH solution to the methanolic extract caused complete decolorization within a few minutes and exposure of the pigment to room light caused complete decolorization within a day.

Chromatograms prepared to test for the presence of alkaloids with Dragendorff's reagent indicated that the alkaloid positive spots did not correspond to the pigment areas. This indicated that these pigments were not alkaloidal in nature.

Hygrophorus psitticinus (Fries) Fries var. psitticinus. Epicrisis Systematis Mycologici. p. 332. 1838.

Tables 24, 37, and 38.

As in Hygrophorus marginatus, the pigments were soluble in the petroleum ether epiphase, and paper chromatography again yielded separations quite different from those of H. flavescens and similar species. A weak salmon colored area which exhibited tailing was found near the origin, while a yellow spot was found at the solvent front (Table 24). An ultraviolet fluorescent area which was colorless was found at $R_f = 0.25$.

Chromatography using Gelman ITLC Type SG sheets developed with

TABLE 23

HYGROPHORUS MARGINATUS var. MARGINATUS & var. CONCOLOR

ACETONE/H_2O 7:3 3 MM PAPER

Band	R_f	Color, Visible	Color, Long UV
1	0.17	mod. orange (tailed)	dull yellow (tailed)
2	0.28	----	pale bluish-white
3	0.35	----	dark area

TABLE 24

HYGROPHORUS PSITTICINUS var. PSITTICINUS

ACETONE/H_2O 7:3 3 MM PAPER

Band	R_f	Color, Visible	Color, Long UV
1	0.0 (tailed,	mod. reddish-orange M (tailed; tailed color decreases uniformly as end of tail is approached)	pale pinkish, becoming yellowish near R_f = 0.11
2	0.25	----	pale bluish-white
3	1.00	moderate yellow S	dark brown

petroleum ether/acetone yielded a yellow band which was separate from a weak salmon colored band. Due to the small amount of available material, this was the only criterion of purity employed with this extract. The absorption curve of this eluted band was again trimodal but the side peaks appeared only as inflections on either side. As shown in Table 37, the maximum of the central peak in chloroform is 454 nm while with methanol as the solvent, the maximum is 446 nm.

After extraction with methanol, the carpophores are somewhat salmon in color. Extraction with pyridine removed most of this coloration and yielded a salmon colored solution. This extract produced a broad absorption curve with a single maximum at 464 nm (pyridine).

Hygrophorus nitidus Berkeley and Curtis. Ann. Mag. Nat. Hist. II (12):424. 1853.

Figures 9 and 10.
Tables 25, 26, 35, and 37.

Unlike either H. marginatus or H. psittacinus, all of the pigments of this fungus remained in the hypophase when the crude extract was washed with petroleum ether. This aspect of H. nitidus was the same as that of the crude pigment extract obtained from H. flavescens, but paper chromatography with an acetone/water mixture as the developing solvent yielded an entirely different pattern (Table 25). Most of the pigment was found between R_f = 0.40 and R_f = 1.00 in what appeared to be three badly tailed and overlapping spots. Under

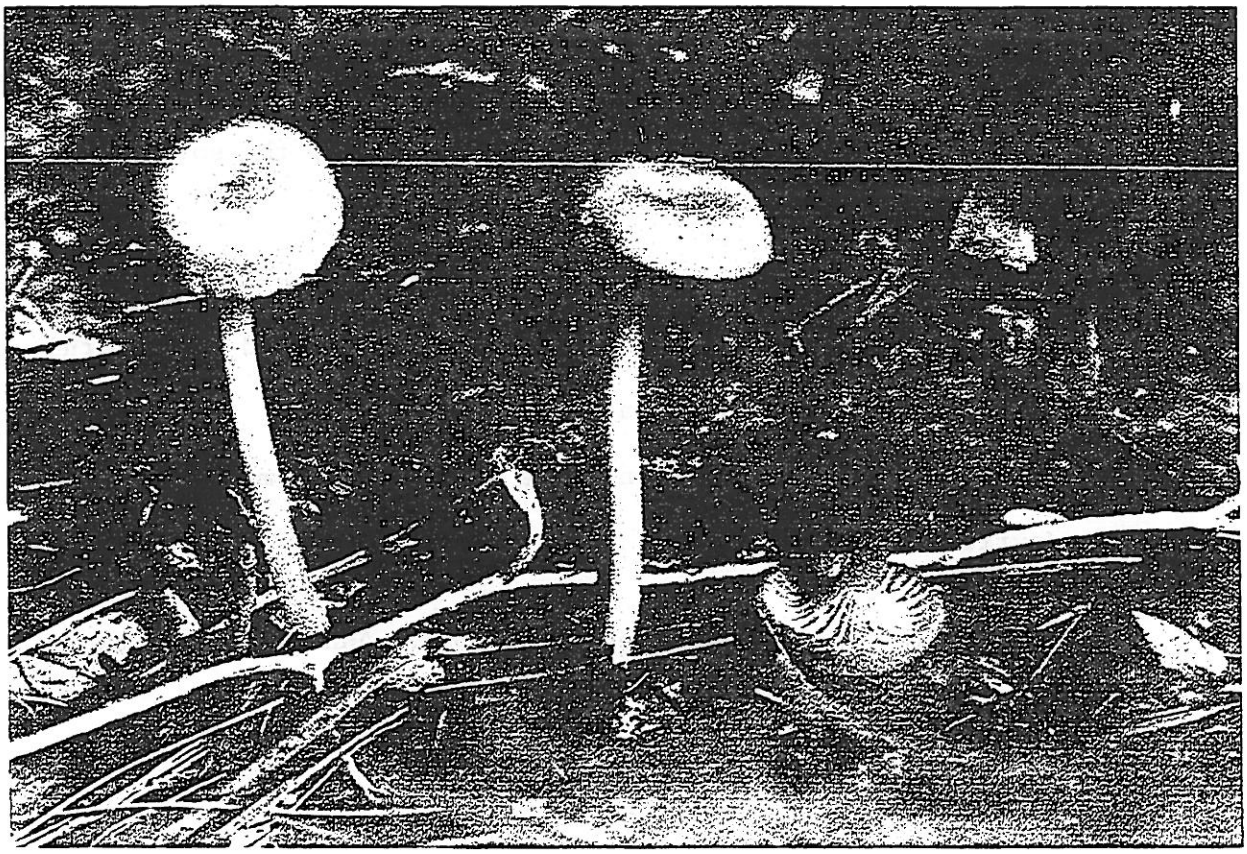

Figure 9. Hygrophorus nitidus. X1.

long wave ultraviolet, all of these spots fluoresced with a dull rust color. When this extract was spot tested for the presence of polyene types, there was an immediate, definite, strong positive reaction. With H_2SO_4, an instant change to purplish-blue is observed: Munsell notation: 7.5PB 2/6 to 7.5PB 2/4. Where the dried pigment was not as concentrated in the depression, a color of 7.5PB 3/4 was determined.

In addition to the routine chromatography attempted using paper, various other solid phases for development also were examined. The use of "PreKote" Silica Gel G plates[3] with BAW 6:1:2 as the developing solvent gave the separations shown in Table 26. Spraying this chromatogram with antimony trichloride gave a positive test for polyenes at the sites of all colored areas. In addition, iodine vapor produced brown areas at these sites as well as at one other site indicating the presence of conjugated double bands (Davies et al., 1963).

Elution of the major band and subsequent spectrophotometry again yielded the trimodal absorption spectra typical of polyene pigments (Figure 10 and Table 37), as well as the spectral shifts expected in solvents of differing polarity with the loss of fine structure most noticeable when water was used as a solvent. The striking feature of this pigment was its solubility in water; this is most unusual for a polyene.

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[3] Available from Applied Science Laboratories, P. O. Box 440, State College, Pa. 16801

TABLE 25

HYGROPHORUS NITIDUS

ACETONE/H_2O 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0	p. yellow	W	brown	M
2	0.09	v.p. yellow	VW	p. orange	W
3	0.13	v.p. yellow?	VW	p. yellow	W
4	0.40	vivid yellow (tailed)	S	rust color	S
5	0.51	vivid yellow (tailed)	S	rust color	S
6	0.79 to 1.00	vivid yellow (tailed)	S	rust color	S

TABLE 26

HYGROPHORUS NITIDUS

BAW 6:1:2, SILICA GEL G PLATES

Band	R_f	Color Visible	Color with I_2 vapor	Color with $SbCl_3$ in $CHCl_3$
1	0	v.p. yellow VW	v.p. brown	v.p. blue
2	0.29	--	brown	--
3	0.51	moderate yellow M	dark brown	deep blue→brown
4	0.72	vivid yellow VS	brown	light greenish-blue
5	0.96	mod. yellow M	brown	light blue

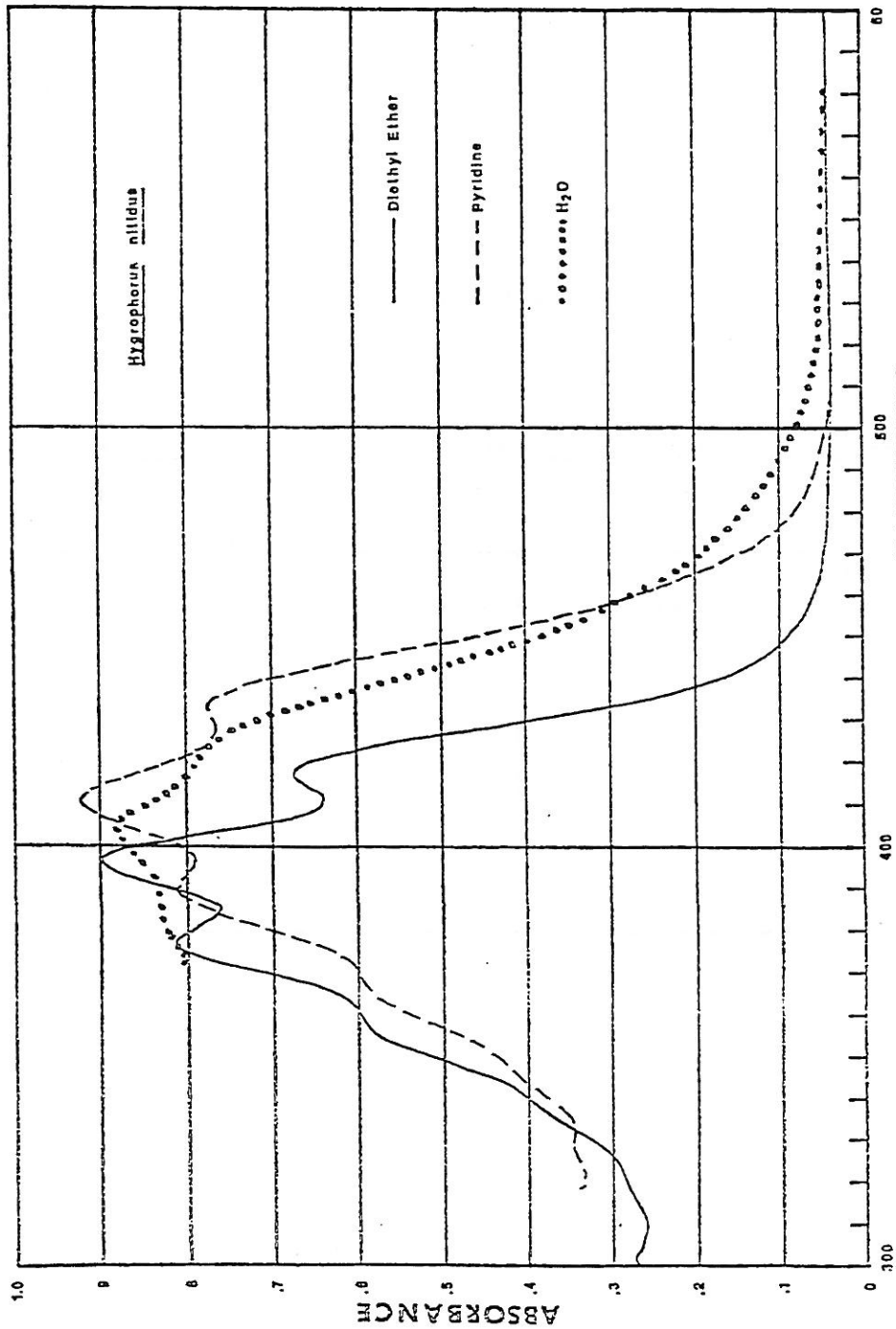

Figure 10. Absorption spectra of the polyene pigment present in *Hygrophorus nitidus*. Spectra were obtained in water, pyridine and diethyl ether. The pigment was insoluble in petroleum ether and other similar, non-polar organic solvents.

Since some of the non-polar pigments are soluble in solvents such as water when in glycosidic form, hydrolysis sometimes can be used to permit these pigments to be taken up in non-polar solvents such as petroleum ether. Hydrolysis, either by acid with refluxing, or enzymatic (glycolytic and proteolytic enzyme treatments) failed to yield aglycone which exhibited a solvent solubility different from that of the untreated extract. Staining the chromatograms with Amido Black (Dawson et al., 1969) did not reveal the presence of protein and indicated that the pigment is not a carotenoprotein. Addition of sodium dithionate did not cause a decolorization of an aqueous solution of this pigment.

Separation by chromatography was the only criterion of purity used in the determination of the visual spectral data reported herein as only small amounts of extract were available. Unfortunately, insufficient material was available for analysis with IR, NMR, or mass spectroscopy.

Hygrophorus pratensis (Fries) Fries var. pratensis. Epicrisis Systematic Mycologici. p. 326. 1838.

Table 35.

This Hygrophorus, which has a dull orange to rufous colored pileus and rather a cantharelloid aspect, was collected in small quantity once during the study. Attempts to remove the pigment from the pileus with methanol and acetone were not successful. In this respect, the pigment does not resemble those found in H. flavescens

and related species. It does not appear that this pigment is a carotenoid as are the pigments found in several species of Cantharellus since this pigment was not extractable with solvents which normally would extract carotenoids from fungal tissue.

Miscellaneous Agarics Studied

Mycena leaiana (Berkeley) Saccardo. Sylloge Fungorum 9:38. 1891.

Tables 27, 28, and 29.

According to Fayod (1889) and Hesler and Smith (1963), the genus Mycena is derived in part from Hygrophorus. In view of this, it seemed appropriate to examine some brightly colored Mycenas which have pigments similar to Hygrophorus.

The pigment of M. leaiana is freely soluble in water and to a lesser extent, in the short-chain more polar alcohols. When this pigment extract was chromatographed there was a separation entirely different from that of H. flavescens and related species as shown in Table 27.

Rhodohygrocybin and flavohygrocybin are absent, and the bulk of the pigment is located in what might be two spots of slightly differing R_f, but both spots are tailed and overlap. However, the separations and ultraviolet fluorescence are very similar to that observed for H. nitidus. As shown in Table 27, the R_f values of the major pigmented compounds were near 0.90. Spot testing the crude extract for the presence of polyene type pigments gave an

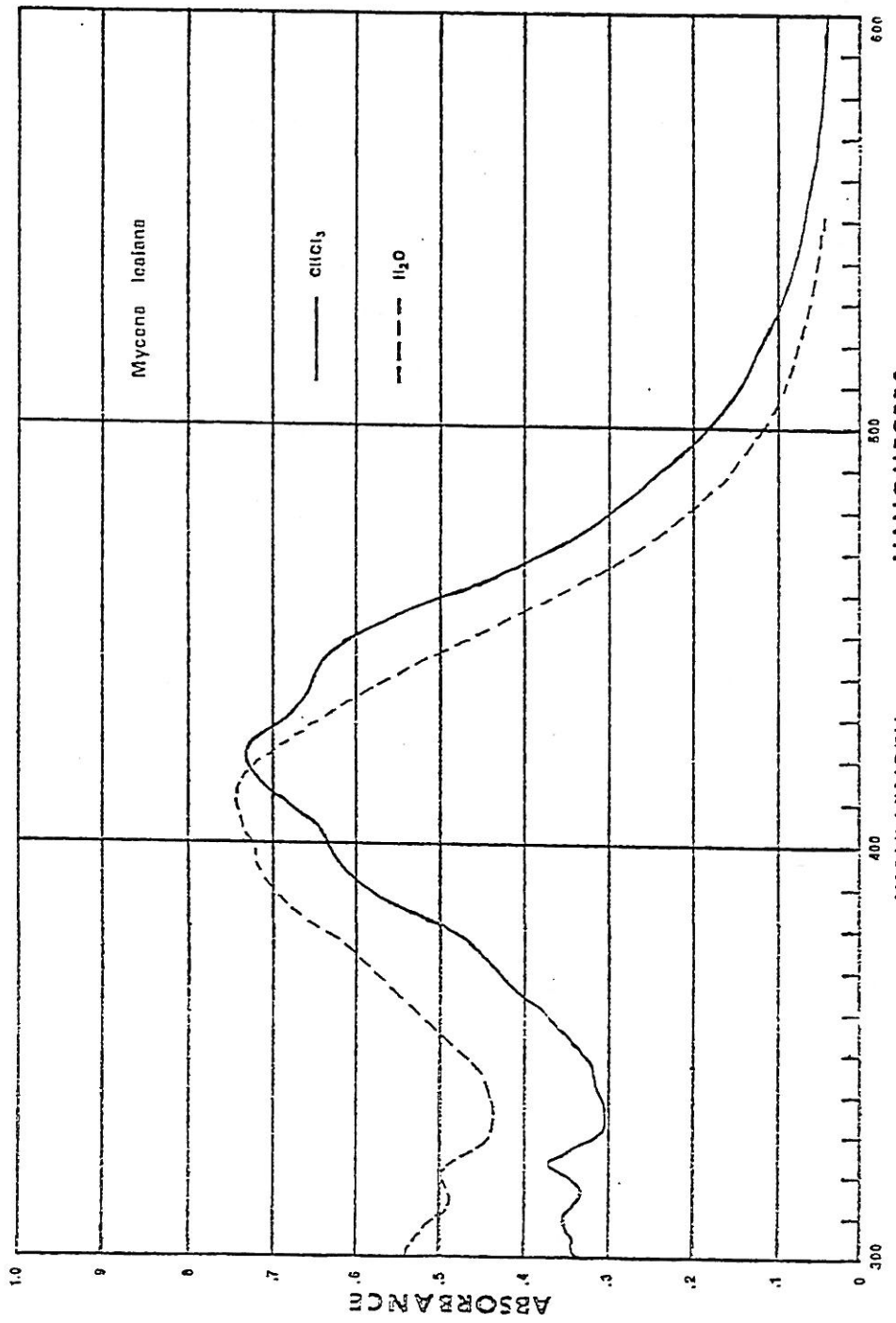

Figure 11. Absorption spectra of the major polyene pigment found in *Mycena leaiana*.

immediate positive response with either sulfuric acid or antimony trichloride.

As with both crude rhodohygrocybin and flavohygrocybin extracts, the crude extract had abundant ninhydrin sensitive materials even after lipids had been removed by evaporation to dryness and the pigments rehydrated with water. As shown for other agarics in Figure 18, the ninhydrin sensitive materials all had R_f values below 0.55. This was also true for Mycena leaiana. However, attempts to remove ninhydrin sensitive materials by ion exchange columns (p. 50) could not be employed as the pigments became bound irreversibly to the column and could not be removed. It was learned that there were two other ways in which the ninhydrin sensitive compounds could be removed. Preparative band chromatography with paper and subsequent elution of the pigmented areas yielded extracts which were now not sensitive to ninhydrin. It was found also that the pigments were moderately soluble in chloroform while ninhydrin sensitive materials are not. The latter method using chloroform was chosen for the subsequent studies.

After removal of lipids (p. 42) the aqueous solution was evaporated under reduced pressure and chloroform was then added. This dissolved most of the pigment but left behind a water-soluble, light yellowish colored residue which was strongly sensitive to ninhydrin.

Subsequent chromatography of the chloroform-soluble fraction yielded the results shown in Table 28. The most intense band with

TABLE 28

MYCENA LEAIANA BAW 6:1:2

Band	R_f	Color, Visible		Color, Long λ UV	
SILICA GEL G PLATES					
1	0.69	vivid yellow	VS	yellow	S
2	0.83	pale yellow	W	pale yellow	W
GELMAN ITLC PLATES, TYPE A					
1	0.81	vivid yellow	VS	yellow	S
GELMAN ITLC PLATES, TYPE S					
1	0.75	mod. yellow	M	yellow	S
2	0.93	----		yellow	W

TABLE 27

MYCENA LEAIANA

ACETONE/H_2O 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.02	--		p. yellow	VW
2	0.16	l. yell. brown	VW	p. yellow	VW
3	0.21	v.p. yellow	VW	p. yellow	VW
4	0.37	--		p. yellow	VW
5	0.67	--		yellow	MW
6	0.90	vivid oy - tailed	S	brilliant yellow tailed	S
7	0.97	vivid oy	S	brilliant yellow	S

R_f = 0.69 found for the silica gel G plates was chosen for further study. Elution of this band and rechromatography with any of the solvent systems discussed here produced only a single spot. This appeared to indicate that a reasonably pure compound was analyzed.

This pigment is an amorphous powder which is a deep reddish-brown (ISCC-NBS #40) in color when kept under vacuum or under dry nitrogen. The pigment is strongly hygroscopic and upon exposure to air soon forms a tarry residue which is also a deep reddish-brown in color.

The major absorption maxima in the visible of this pigment are shown in Figure 11 and in Table 37. Additionally, absorption was also found in the ultraviolet; peaks at 310 and 323 nm in chloroform and peaks at 282, 300, and 319 in water were also observed.

Addition of sodium dithionite ($Na_2S_2O_4$) did not cause decolorization and the addition of aqueous potassium hydroxide did not cause a spectral shift in the absorption curve.

A mass spectrum of the purified pigment was performed in the Mass Spectrometry Laboratory, Department of Chemistry, University of Massachusetts. This analysis yielded M+ = 280 and the complete spectrum with the operating conditions is shown in Figure 12. NMR spectroscopy was attempted with the pigment dissolved in deuterated chloroform, but even with a saturated solution, a spectrum could not be obtained as this solution was still much too dilute. What is needed for studies such as these is an NMR unit which could repeatedly scan the sample, storing the signal from each scan and then printing

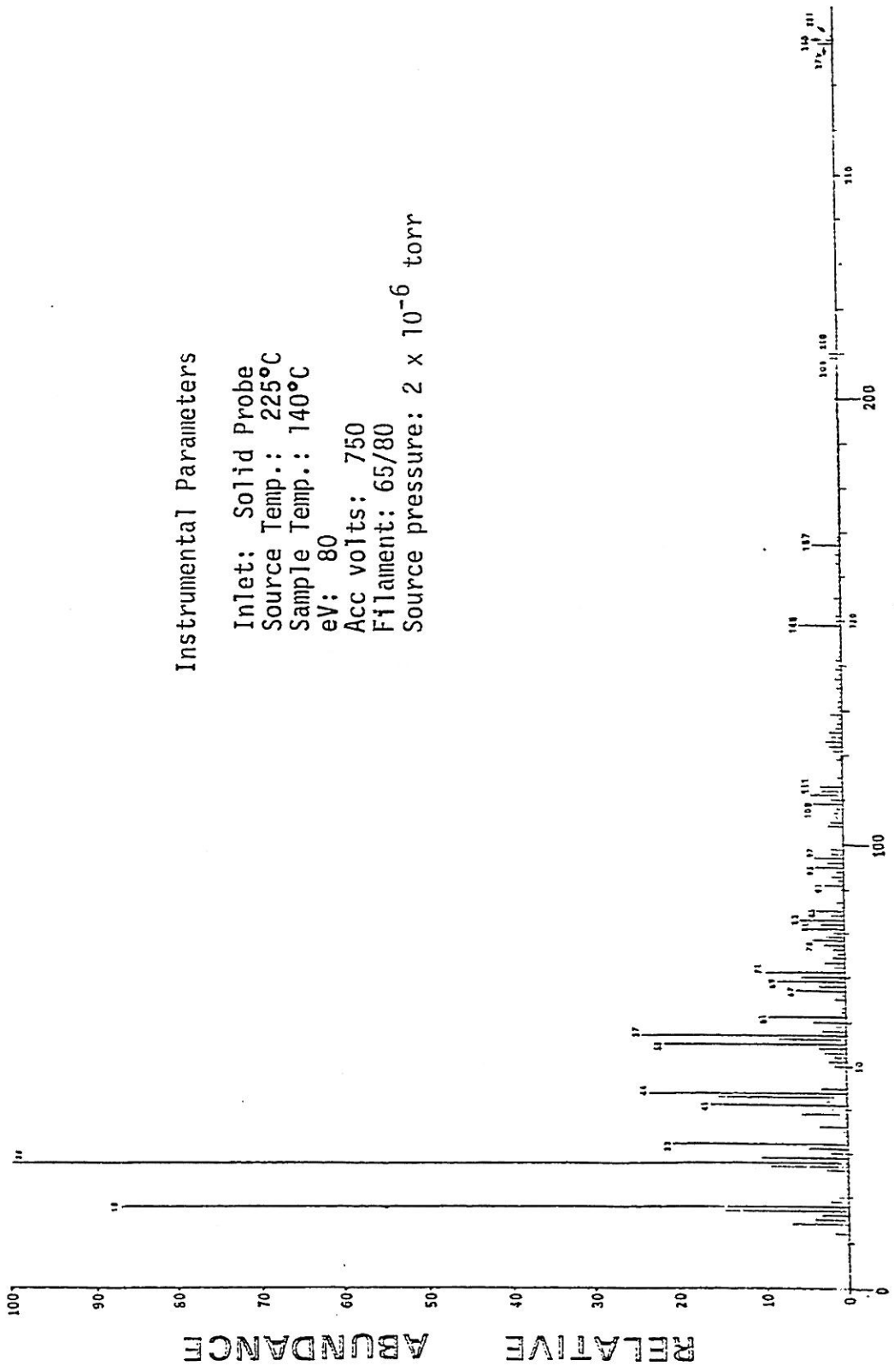

Figure 12. Mass spectrum of major pigment found in *Myeena leaiana*.

a spectrum which would be the integrated result of many scans.

Infrared spectroscopy of a dilute solution of this pigment in chloroform, produced a spectrum with limited information. The discernable absorption peaks are shown in Table 29.

TABLE 29

INFRARED ABSORPTION MAXIMA FOR

MAJOR PIGMENT FOUND IN

Mycena leaiana

Wavelength	Absorption	Wavelength	Absorption
3030 cm^{-1}	W	1218 cm^{-1}	S
2930	W	1015	W
1730	W	892	S
1640	VW	730	W
		723	W

Mycena epipterygia (Fries) Kummer var. cespitosa Thiers
Mycologia 50:517. 1958.

Table 37.

This densely cespitose Mycena was collected once in De Soto National Forest, Mississippi. By its lignicolous and cespitose habit, it resembles M. leaiana, but is much more muted in color and more yellowish.

Extraction of the sporophores with methanol yields a clear lemon yellow extract, leaving the sporophores with a pallid brown coloration. This extract gives a positive test for polyenes, and spectrophotometry of the crude extract yields an absorption curve with the trimodal aspect expected of a polyene. In methanol, the absorption maxima were: 376, 392, and 410 nm.

Mycena amabilissima (Peck) Saccardo. Syll. Fung. 9:37. 1891.

Table 35.

This one collection studied was found in association with Sphagnum and Ledum groenlandicum (Labrador tea), in much the same habit as Hygrophorus turundus. This collection was of interest since Hesler and Smith (1963) postulated a possible phylogenetic relationship between subsection Hygrocybe of Hygrophorus and section Adonidae of Mycena. M. amabilissima is a representative of section Adonidae of Mycena. In part, this theory is based upon the bright colors found in many species of section Adonidae.

As found in some Hygrophori already examined, M. amabilissima has reddish colors and is differentiated from Mycena adonis principally on the basis of the color changes produced in the sporophores. In M. amabilissima, the brilliant pinkish fades to lighter shades without fading to yellow as with M. adonis.

The pigments present in M. amabilissima were not extractable by either polar or non-polar solvents unlike those pigments studied in Hygrocybe. Due to limited material, further investigations on

M. amabilissima were not continued at this time. Although this Mycena is unlike Hygrocybe by not possessing an extractable pigment and not being yellow in pigmentation, there is the distinct possibility that the observed yellow pigment found in M. adonis which remains after the red has faded is similar or identical to flavohygrocybin.

Mycena pura (Fries) Quelet. Champ. Jura et Vosges, p. 103. 1872.

Figure 14.
Table 35.

As this Mycena exhibits a purplish color in the field and is placed in the section Adonidae, it was examined for the presence of flavohygrocybin or rhodohygrocybin. Paper chromatography (acetone/water, 7:3) showed a weak greyish-purple spot at R_f = 0.26 which appeared dark under long wave ultraviolet. No other spots were observed. Spectrophotometry gave absorption maxima at 243, 360, 538, and 758 nm. The very broad absorption maximum at 538 nm is the least intense. A second collection (393) showed two peaks in the visible at 482 and 513 nm thus differing from the earlier collection. These differing results are not surprising as Smith (1947) comments on the extreme variability in color of this Mycena.

Chromatography and spectrophotometry both demonstrate that neither flavohygrocybin nor rhodohygrocybin were present. Also, in the visible, the absorption is totally dissimilar when compared to the absorption noted for Mycenas which have polyene pigments (e.g. M. leaiana). Similar negative results were also found with the other

species listed in Table 35 and the procedures described here serve as an example of the determination of these other negative results.

Cantharellus cinnabarinus (Schweinitz) Schweinitz. Naturf. Gesell. Leipzig Schrift. 1:83. 1822.

Since this Cantharellus is known to contain canthaxanthin (Structure XXXIII) as its primary pigment (Haxo, 1950), it was examined using the spot test for polyenes (positive) and the crude extract was processed as outlined by Cooney et al. (1966) to isolate canthaxanthin.

Spectrophotometry of the eluted pigment yielded λ_{max} = 467 nm in ethanol and λ_{max} = 498 nm in carbon disulfide. This is in good agreement with published absorption maxima (Davies, 1965) for this carotenoid.

Craterellus cornucopioides Fries. Epicrisis Systematis Mycologici. p. 532. 1838.

This fungus is considered to belong in the Cantharellaceae, and had also been studied with respect to pigmentation by Fiasson (1968). A collection was made to evaluate the pigments of this species in light of the data now available on Hygrophorus and also to compare the data on American collections of this Cantharellus with the data of Fiasson for European material.

The pigments extracted well with MeOH, and after extraction, with the addition of petroleum ether and water, the pigments were found in the epiphase. Spectrophotometry of the crude extract gave

absorption maxima at 416, 442, 469, and 500 nm; nearly identical to the data recorded by Fiasson. He has shown that these absorption maxima result from neurosporene (60%) and lycopene (40%) respectively.

Amanita flavoconia Atkinson. Jour. Myc. 8:110. 1902.

Table 30.

Singer (1951) considers a relationship between Amanita and Hygrophorus a possibility, with species of Biannularia G. Beck (= Catathlesma Lovejoy) and Armillaria (Fries) Quélet as possible intermediates. Although most species of Amanita are white or dull colored, several species are notable for their brilliant pigmentation. Possibly the latter might have pigments similar to those found in Hygrocybe, although there are other sections in Hygrophorus with which Amanita could be connected.

Amanita flavoconia is one of three brightly colored Amanitas which were studied and the solvent systems that proved suitable for the hygrophoroid pigments also gave excellent separations here.

As with the pigments in section Hygrocybe, the Amanita pigments were quite labile and best studied with absolutely fresh material. The extractions and chromatography worked best when performed under an inert atmosphere of nitrogen. The results are given in Table 30.

The band at R_f = 0.47 is suggestive of flavohygrocybin! When eluted and co-chromatographed with flavohygrocybin isolated from Hygrophorus miniatus var. miniatus, only one spot results and this

TABLE 30

AMANITA FLAVOCONIA

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV
1	0.10			dark band
2	0.32	orange-yellow	S	yellowish
3	0.38	brill. yellow	M	green-yellow
4	0.47*	brill. yellow	M	green-yellow
5	0.60	p. yellow	W	v.p. whitish
6	0.72	---	-	bluish
7	0.77	---	-	bl. white

pigment exhibits an absorption maximum at 420 nm, all indicating an identity with flavohygrocybin. The presence of flavohygrocybin gives additional weight to Singer's suggestion.

Amanita muscaria (Fries) S. F. Gray. Nat. Ann. Brit. Pl. 1:600. 1821.

Tables 31 and 32.

As noted previously (p. 31 to 36), the pigments of the fly agaric have been studied more intensively than any others in agarics, but only very recently have their unique chemical structures been partially eludicated.

Paper chromatography of the yellow to yellowish-orange variety found in the northeast yielded seven separate, identifiable pigments (Table 31). Talbot and Vining (1963) found only three pigments in their analysis, while Döpp et al. (1971) identified four chromophoric agents. Later Döpp and Musso (1973) listed nine separate identifiable pigments as being present in European collections. However, it must be emphasized that the deep red variety commonly found in Europe is extremely rare in the northeastern states. My collections were closer in color to those reported by Talbot and Vining.

The pigment at R_f = 0.50 appears to be similar to flavohygrocybin by exhibiting a chartreuse fluorescence under long wave ultraviolet and by having a visual absorption maximum at 420 nm, but the R_f value is somewhat high for flavohygrocybin in this solvent system.

TABLE 31

AMANITA MUSCARIA, YELLOW FORM

ACETONE/WATER 7:3 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.00	--		bright bl. white	S
2	0.37	p. orange-yellow	S	p. yellow	W
3	0.41	m. orange-yellow	M	p. yellow	W
4	0.45	m. orange-yellow	M	p. yellow	W
5	0.50	p. yellow	W	p. green-yellow	W
6	0.54	p. yellow	W	p. green-yellow	W
7	0.57	p. yellow	W	p. bl. green	W
8	0.61	v.p. yellow	VW	p. lt. blue	W
9	0.65	--		bl. white	M

TABLE 32

AMANITA MUSCARIA, RED FORM

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible	Color, Long λ UV	
1	0.22	p. orange-yellow	yellow-orange	M
2	0.27	p. yellow	bluish-white	W
3	0.31	p. yellow	salmon	W
4	0.51**	p. yellow	chartreuse	M

It would be desirable to examine fresh material of European specimens to determine what separations would be obtained from these deeply red colored sporophores with the chromatographic systems used in this study. This is of particular interest since Döpp et al. (1971) report the presence of a pigment with an absorption maximum of 540 nm. The presence of such a pigment was not demonstrated in the New England collections.

A deeply red colored form of this agaric does occur in quantity along the Gulf Coast during the winter. Although it is not known whether this form is identical with European material, the separations (Table 32) indicate the presence of a pigment which on the additional basis of electronic absorption spectra, appears to be identical to flavohygrocybin. This is significant in respect to the recent studies of Döpp and Musso (1973a) who propose a dihydropyridine structure for a similar compound (structure XL), while somewhat later, these same authors (1973b) as well as Besl et al. (1975) suggest a dihydroazepin structure for this same pigment (XLIV).

Amanita caesarea (Fries) Schweinitz.

Table 33.

This Amanita, considered to be related to Amanita muscaria, also has a pigment (R_f = 0.51) which appears to be identical to flavohygrocybin as shown in Table 33. In addition, one band (R_f = 0.26) present in small amount, appears to be similar to rhodohygrocybin,

TABLE 33

AMANITA CAESAREA

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0.22	yellow	W	yellow	W
2	0.25	orange	M	yellowish-brown	M
3	0.26	reddish	W	pale rose	W
4	0.28	orange-yellow	W	bluish-white	W
5	0.33	yellow	W	greyish-brown	W
6	0.51**	vivid yellow	S	bright chartreuse	S

but spectroscopic data is not available on this compound.

Lepiota lutea (Bolton) Quélet

Table 35.

This delicate, pale yellow pubescent Lepiota is common along the Gulf Coast in grassy areas during the hot, rainy weather of August and September. Because of its color and as a representative of another white-spored family, the Lepiotaceae, I was prompted to examine this species in more detail.

The pigment is readily extractable in methanol, and spectrophotometry shows a single broad absorption maximum in the ultraviolet at 373 nm. The visible color of this plant simply results from the long wavelength falloff which extends into the visible.

A methanolic solution of this pigment is strongly fluorescent under long wavelength ultraviolet, and the dried sporophores exhibit a beautiful yellow fluorescence under the same radiation. The extract gives a negative test for polyene pigments.

Russula veternosa Fries. Epicrisis Systematis Mycologici. 1838.

Figures 17 and 18
Tables 34 and 35.

Recent investigations of Eugster and Frauenfelder (1970) and Iten et al. (1973), have shown that one of the primary pigments in many red species of Russulas is Russupteridin-S_{III}. Much earlier, Zopf (1890) indicated that a similarity existed between the pigments

present in species of Hygrophorus clustered around H. coccineus and various red species of Russula. This suggested that the techniques which were effective for the analysis of Hygrophorus pigments might also be applied to a representative collection of a red Russula in order to determine if Russupteridin-S_{III} is similar or identical to rhodohygrocybin.

Two good collections of Russula veternosa were obtained, and the pellicle containing the pigments was carefully peeled from the sporophores and was then extracted with pyridine. This solvent effectively removed the pigment from the pellicles. The crude extract obtained in this manner accounted for approximately 0.4% of the whole weight of the sporophores. Chromatography of this crude extract yielded good separation of the pigments as shown in Figures 17 and 18, and in Table 34.

As shown in Figures 17 and 18, the amount of ninhydrin sensitive compounds extracted from this Russula is considerably less than that extracted from the Hygrophori, probably as a result of the use of pyridine as an extractive solvent. Also, by virtue of its low R_f with this solvent system, Russupteridin-S_{III} is free of these compounds (Figure 18).

Russupteridin-S_{III} was effectively removed from the chromatogram with pyridine as the elutant. After the solvent was evaporated, the pigment remained as a reddish-black amorphous, hygroscopic powder. Spectrophotometry of this pigment in pyridine gave absorption maxima at 562, 524 infl., 396, and 324 nm. The principal

TABLE 34

RUSSULA VETERNOSA

ACETONE/WATER 6:4 3 MM PAPER

Band	R_f	Color, Visible		Color, Long λ UV	
1	0	lt. grey		bluish	W
2	0.09	strong purplish red	VS	brill. orange	VVS
3	0.39	-		s. blue-green	S
4	0.55	-		bright blue	S
5	0.65	-		p. yellow	W
6	0.78	-		p. yellow	W
7	1.00	p. brownish	W	bright p. yellow	M

absorption peak at 562 nm is a much sharper peak than the absorption maximum found with either rhodohygrocybin or flavohygrocybin, and agrees well with the data reported by Eugster (1970). This is especially noteworthy since the techniques used by Eugster were quite sophisticated (isoelectric focussing with a pH sucrose gradient) and may not be available in many laboratories.

Russupteridin-S_{III} appeared to be much more stable than the pigments isolated from Hygrocybe; the brilliant magenta spots of Russupteridin-S_{III} are still present twenty-eight months after preparation of the chromatograms, while the pigments separated from species of Hygrocybe faded within a month. These differences indicate that Russupteridin-S_{III} is not identical with rhodohygrocybin.

In addition to the agarics discussed above, a variety of other fungi were evaluated with the methods used in this study. These all lacked polyenes, flavohygrocybin and rhodohygrocybin and are listed in Table 35.

Identification of Pigments

Pigment topography.

Microscopic examination of the hyphal components of the sporophores of many species of Hygrophorus section Hygrocybe, discloses that the hyphae vary from hyaline to deeply colored. The hyphae which are colored possess vacuolar pigments in contrast to the granular nature of the pigments observed in some species of Cantharellus or encrusting pigments found in some species of

TABLE 35

EUMYCOTA WHICH DO NOT HAVE POLYENES,

FLAVOHYGROCYBIN OR RHODOHYGROCYBIN

AS PIGMENTS

Hygrophoraceae

Hygrophorus pratensis (Fries) Fries, H. subviolaceous Peck, H. flavodiscus Frost apud Peck, H. borealis Peck, H. speciosus Peck, H. calyptraeformis Berkley and Broome apud Berkeley.

Lepiotaceae

Lepiota lutea (Bolton) Quelet.

Tricholomataceae

Clitocybe aurantiaca (Fries) Studer, Mycena haematopus (Fries) Quelet, M. pura (Fries) Quelet, M. amabilissima (Peck) Saccardo. Tricholoma flavovirens (Fries) Lundell.

Russulaceae

Russula lutea (Hudson ex Fries) S. F. Gray, R. veternosa Fries, R. flavida Frost and Peck, Lactarius thyinos Smith.

Rhodophyllaceae

Entoloma salmoneum (Peck) Saccardo.

Cortinariaceae

Cortinarius sanguineus Fries, C. semisanguineus Fries.

Strophariaceae

Naematoloma fasciculare (Hudson ex Fries) Karsten.

Cantharellaceae

Gomphus floccosus (Schweinitz) Singer.

Clavariaceae

Ramaria formosa (Fries) Quelet.

Polyporaceae

Polyporus sulphureus Fries.
P. cinnabarinus Fries.

Ascomycetes

Hypomyces lactifluorum (Schweinitz) Tulasne on Russula Sp.

Craterellus. Furthermore, in Hygrocybe the pigmented hyphae are less numerous than the hyaline hyphae and are usually hyphae which constitute the epicutis of the pileus. The hyphae of the context are most often hyaline. Figure 13 illustrates the vacuolar nature of the pigments in the cuticular hyphae of Hygrophorus #485. Additionally, hyphae which clothe the stipe longitudinally are often highly pigmented, although this condition varies among species. Here also, the pigmentation is vacuolar. In certain species (e.g., H. purpureofolius) the lamellae may be deeply colored. In these species, hyphae which compose the gill trama are the hyphae which are pigmented, and the basidia are hyaline.

The probable importance of pigment topography in investigations of agaric taxonomy and phylogeny was first proposed by Kühner (1934). In this study Kühner postulated that pigments which had different cytological distributions represented probable evidence of different chemical composition. These differing cytological dispositions thus indicated the phylogenetic or taxonomic separation of two species with similar coloration.

Chromatography and purification of flavohygrocybin and rhodohygrocybin

It became apparent after preliminary screening and chromatography that a number of Hygrophori in the section Hygrocybe had pigments which appeared identical and which were shared among a variety of species within this section. Figure 14 illustrates four species of

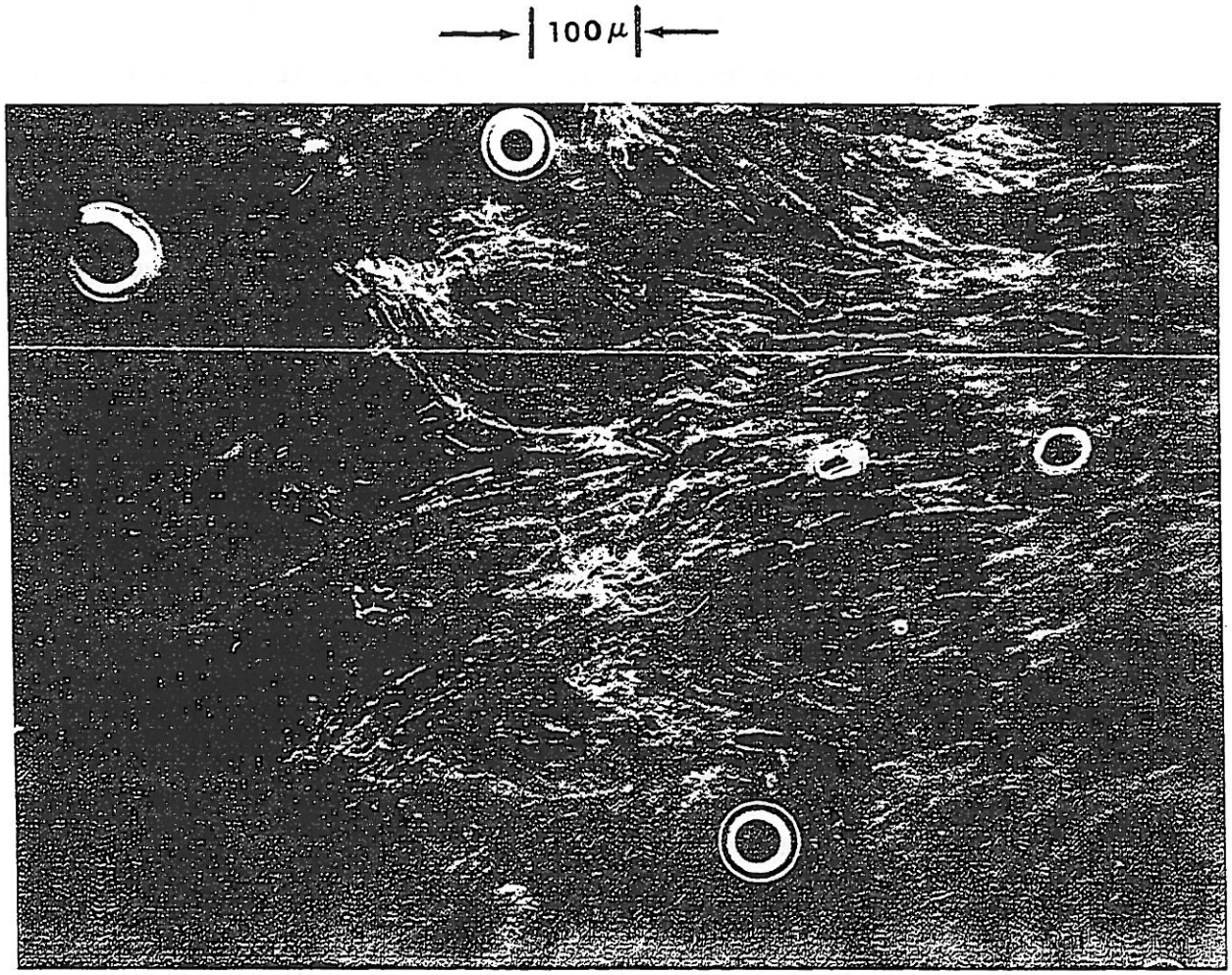

Figure 13. Pigment topography in Hygrophorus (Hygrocybe). Pigmented hyphae found in the pileus epicutis of Hygrophorus #485.

Hygrocybe which have a vivid yellow pigment (R_f = 0.40-0.47, acetone/water 7:3). Since this pigment appeared to be unknown, it is designated here as "flavohygrocybin." In addition, it is evident from the figure that varying amounts of a magenta colored pigment are also found in most of these same species. This pigment, of lower R_f value (R_f = 0.10-0.15, acetone/water 7:3) is named "rhodohygrocybin." Flavohygrocybin yields a striking chartreuse fluorescence when viewed under long-wave ultraviolet light. Figure 15 shows the appearance of this same chromatogram when irradiated with ultraviolet light.

Common organic solvents which had been used with success to isolate and purify many other basidiomycete pigments were tested and found to be unsuitable with species of Hygrophorus. Early in this study it was determined that the pigments of H. flavescens and H. miniatus were freely soluble in water, but insoluble in acetone or 2-propanol. Since both of these solvents are miscible with water in all proportions, the solubility behavior of these pigments suggested that an acetone/water or 2-propanol/water mixture might yield a useful solvent system. Both were successful in that rhodohygrocybin was separated from flavohygrocybin, but the acetone/water solvent system proved to yield better separations than those obtained with the 2-propanol/water solvent system.

As mentioned previously, chromatography with acetone/water as a developing solvent caused variations in R_f values unless the conditions were rigidly controlled. Even under these conditions Ganshirt

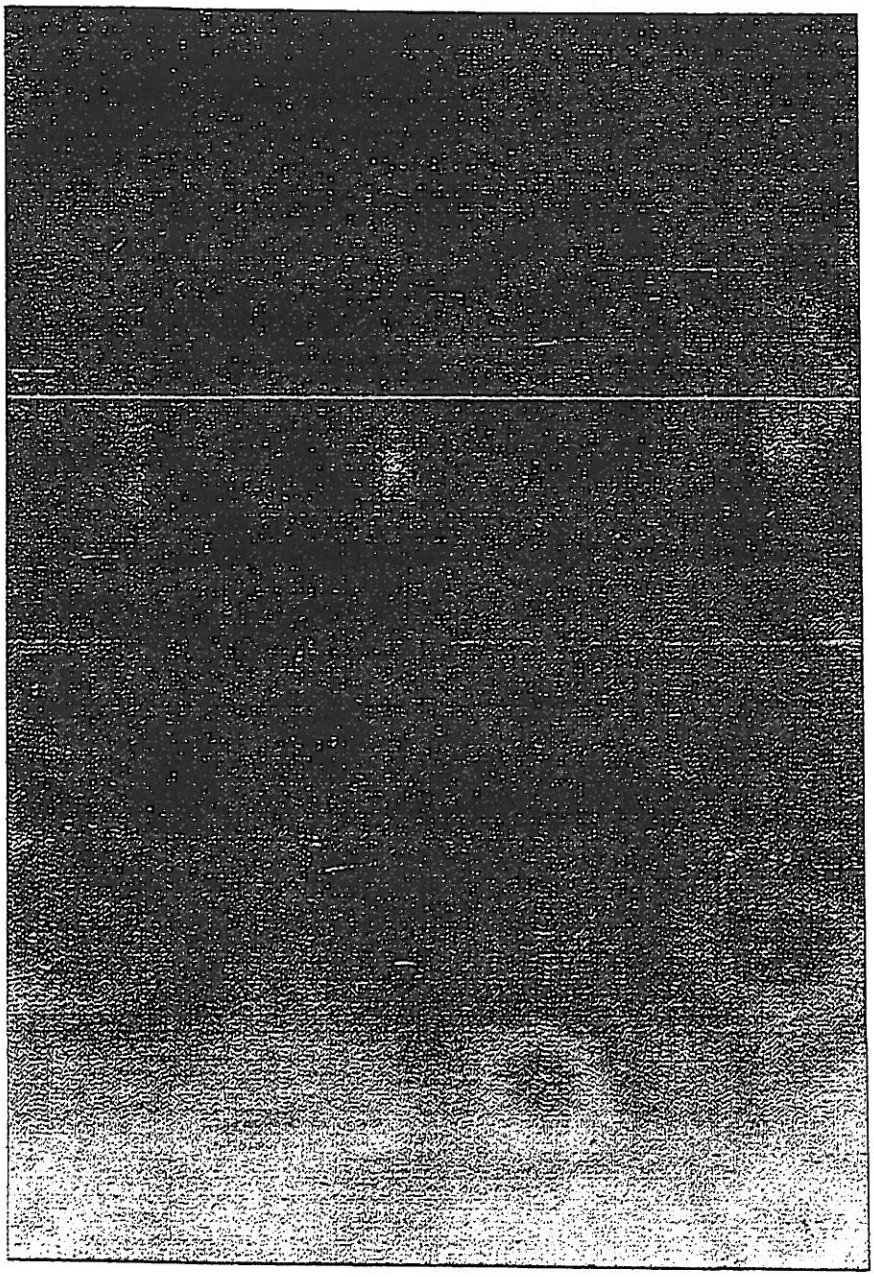

Figure 15. Chromatogram of Hygrophorus pigment, acetone/water 7:3, photographed while illuminated with long wavelength ultraviolet radiation to show fluorescence. A Kodak Wratten HF-2 filter was used to prevent the ultraviolet radiation from reaching the film. Compare the difference between #2 and #6. See also preceding figure.

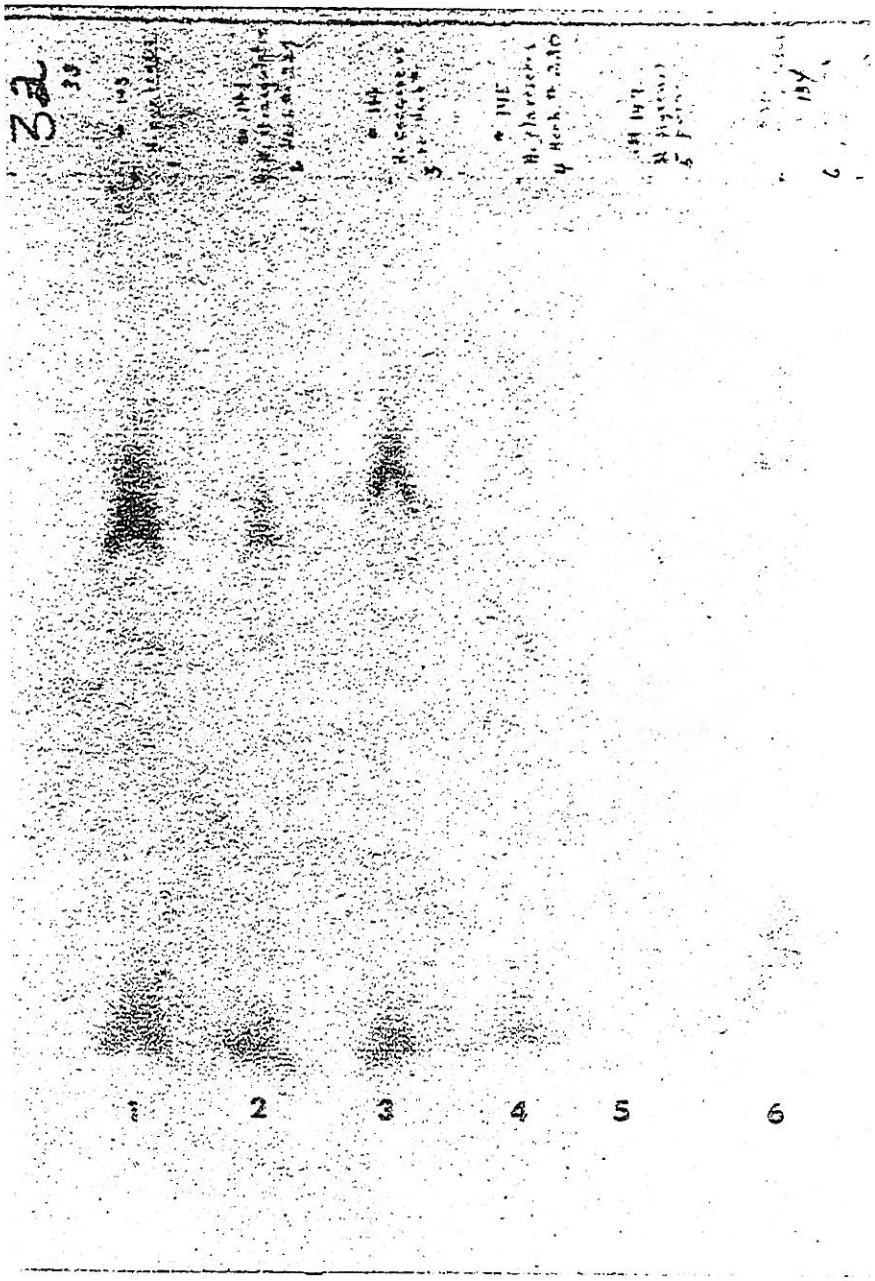

Figure 14. Chromatogram of Hygrophorus pigments, acetone/water 7:3, 3 MM paper. From left to right, the extracts spotted were: 1) Hygrophorus puniceus, 2) H. strangulatus, freshly prepared extract. Compare this with #6. 3) H. coccineus, 4) H. flavescens, 5) Mycena pura, and 6) H. strangulatus, prepared from an extract which stood under room conditions in a lightly covered flask for several days.

(1969) writes speaking of R_f values:

> "The HR_f[4] values are to be regarded only as a guide value for the migration distances, even when all experimental data are accurately measured. Factors like layer thickness (TLC) chamber saturation, air humidity separation effects, can exert a marked influence."

Considerable variation was noted with different runs using the same ratio of acetone/water. Initial separations were obtained using acetone/water ratios of 7:3, but ratios of 6:4 were used later since these ratios appeared to give separations which were somewhat more reproducible. Figure 16 plots the results of a number of runs with differing ratios of acetone/water using crude extracts from H. miniatus f. miniatus at various times. It is evident from this information that with acetone/water ratios which give maximum separation of rhodohygrocybin and flavohygrocybin, small changes in the acetone/water ratio cause large changes in the observed R_f values of these two pigments. Ganshirt, recognizing that studies often are accomplished at different periods in time and perhaps with different chambers, also observes:

> "It is then not useful to measure HR_f values alone, but to compare the migration distances of the substances concerned with that of a simultaneously chromatographed reference substance; this last named should belong to the same of a similar compound class as far as possible."

Flavohygrocybin and rhodohygrocybin were found to be present in both H. flavescens and H. miniatus f. miniatus early in this study. Since these two species were found much more often than the other species

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[4] $HR_f = R_f \times 100$

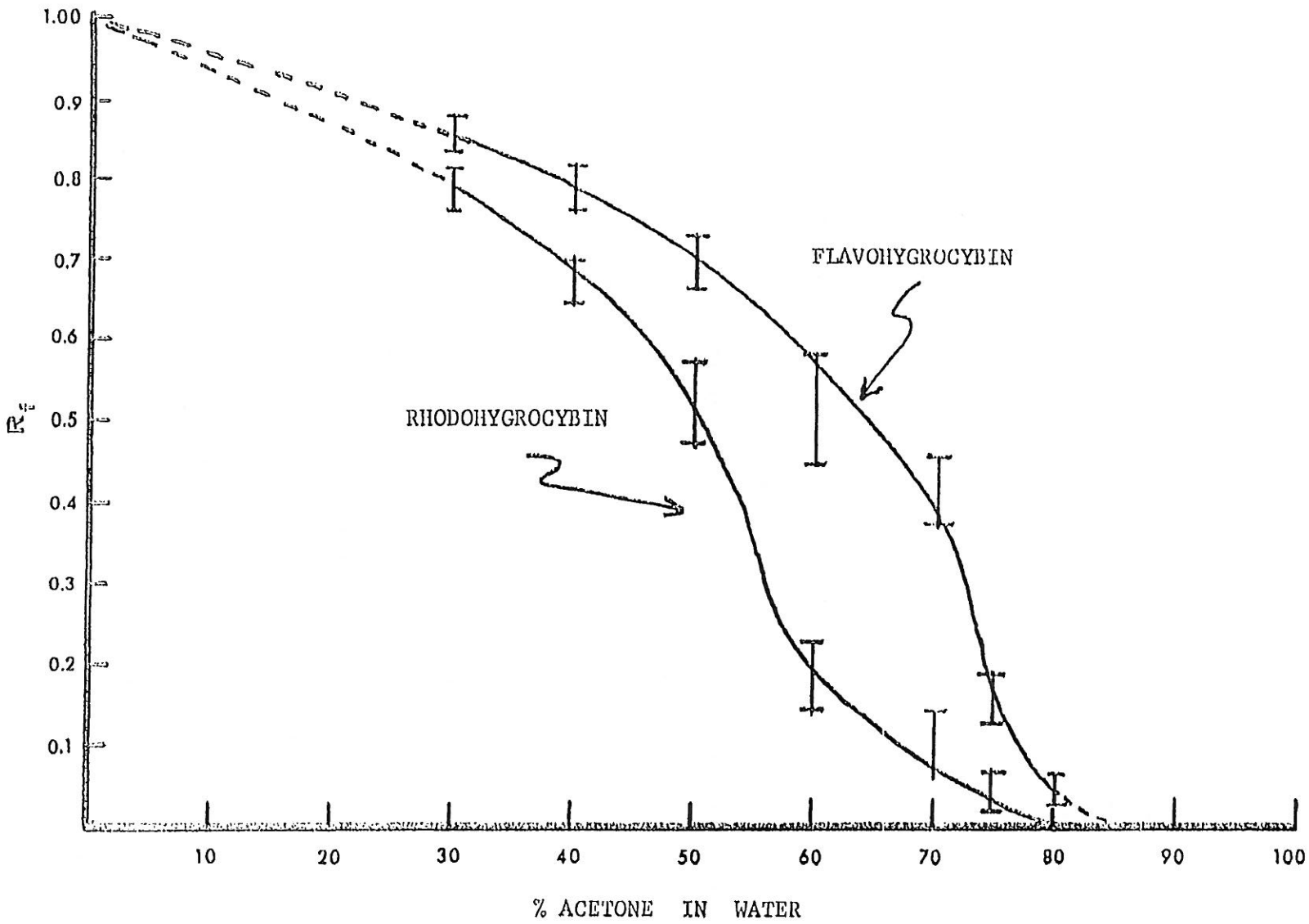

Figure 16. R_f values of flavohygrocybin and rhodohygrocybin using developing solvents with varying ratios of acetone/water.

collected, either was used as a standard by spotting its extract on the chromatogram sheet next to the other extracts. Similar R_f values and identical ultraviolet fluorescence patterns between flavohygrocybin from a reference species and the bright yellow fraction from a test species gave presumptive identity. Spectrophotometry of the eluted fraction from the species under investigation and co-chromatography with flavohygrocybin, confirmed identity of this pigment as flavohygrocybin.

Spectrophotometry using eluates from the band of flavohygrocybin cut from preparative chromatograms early in this investigation showed an absorption maximum in the visible at 420 nm and two in the ultraviolet, one at 258 nm and a second between 202 and 214 nm. The ratio of absorptions of these bands from differing eluates was not constant and led to speculation that the eluted band containing flavohygrocybin might have been contaminated by some other compound. From tests on the bands with Amido black it was known that these chromatographic separations did not contain protein at the sites of pigment separation (Dawson et al., 1969). Representative chromatograms of the crude extracts were then sprayed with ninhydrin (Sigma NIN-3) to examine for amino acids, amines or amino sugars (Brenner et al., 1969). Two such chromatograms are shown in Figures 17 and 18. Figure 18 is the appearance of the separated extracts after spraying with ninhydrin and heating to develop the color. It is evident that in addition to the pigments which were separated that there are many areas which were ninhydrin positive

Figure 17. Chromatogram of various agaric pigments, acetone/H_2O 6:4, 3 MM paper. Reading from left to right, the extracts spotted were: 1) eluate from a band chromatogram prepared earlier from an extract of H. miniatus f. longipes to isolate flavohygrocybin, 2) H. flavescens; 3 and 4) Russula veternosa; 5) H. miniatus f. longipes, fresh, crude extract.

Figure 18. Chromatogram of various agaric pigments, acetone/H_2O 6:4, 3 MM paper, sprayed with ninhydrin. Reading from left to right, the extracts spotted were: 1) eluate from a band chromatogram prepared earlier from an extract of H. miniatus f. longipes to isolate flavohygrocybin, 2) H. flavescens; 3 and 4) Russula veternosa; 5) H. miniatus f. longipes, fresh, crude extract.

including a sample of isolated flavohygrocybin. This suggested that the variable absorption peaks in the ultraviolet from isolated flavohygrocybin might result from a contamination with another amino compound. These ultraviolet absorption peaks at 200-214 and 258 nm were removed through the use of an ion exchange column as described on p. 52-57 and the absorption spectrum appeared as shown then in Figure 19.

It is known that of the 20 common amino acids, three amino acids, namely tyrosine, tryptophan and phenylalanine, absorb significantly in the ultraviolet (Lehninger, 1970 and Sober et al., 1970). Since the absorption maximum of phenylalanine corresponds with the absorption maximum observed at 258 nm, it is probable that phenylalanine is the contaminant having the same R_f as flavohygrocybin. This amino acid was then removed by passage of the extract through a cation exchange column.

The high solubility of flavohygrocybin and rhodohygrocybin in very polar solvents suggested that perhaps these pigments were glycosidic in nature. Representative chromatograms were sprayed with an aniline diphenylamine spray reagent (Sigma ADA-2) to test for the presence of carbohydrates. A positive test is indicated by the appearance of orange or red colors. After treatment with this reagent, a change in color from yellow to orange was noted for flavohygrocybin, suggesting the possibility that flavohygrocybin exists as a glycoside. It should be noted that a positive test could result from the condensation of aniline with betalamic acid

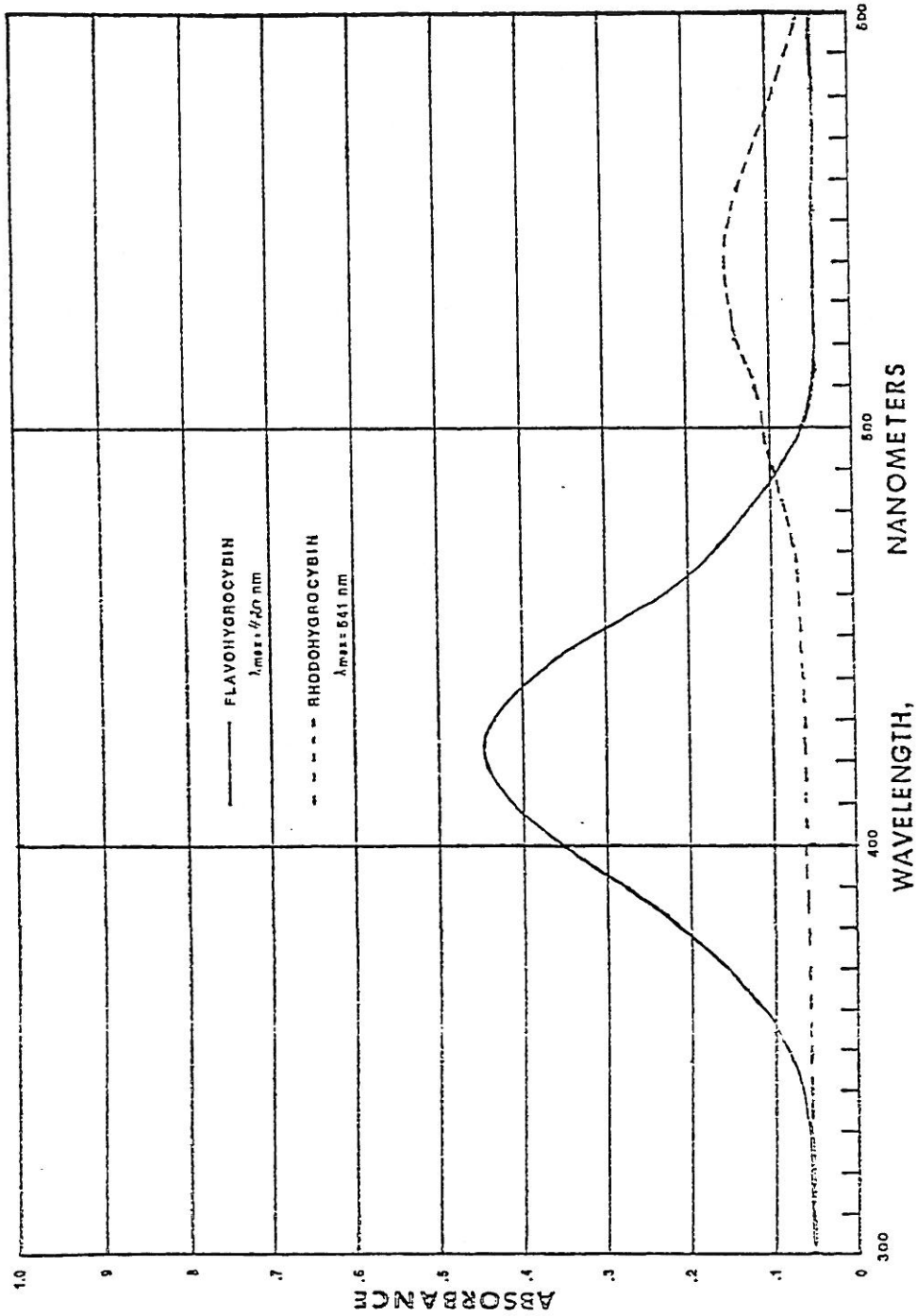

Figure 19. Absorption spectrum of flavohygrocybin and rhodohygrocybin. For flavohygrocybin there is no ultraviolet maximum; the pigment is transparent down to approximately 230 nm where the absorbance gradually increases as 200 nm is reached.

or a compound similar in behavior to betalamic acid. Kimler (1972) describes a series of color reactions to test for the presence of betalamic acid. The use of these reagents produce the expected colors with flavohygrocybin which for aniline, is salmon in color. No color change was observed for rhodohygrocybin, but this is not necessarily a negative reaction since the magenta coloration of this pigment could very well mask an orange or red color change caused by the aniline diphenylamine spray reagent.

Acid hydrolysis of flavohygrocybin was attempted using hydrochloric acid refluxing, but later addition of iso-amyl alcohol or petroleum ether to form a two phase system did not yield separation of a chromophoric aglycone in the epiphase. All coloration remaining after hydrolysis was still found in the hypophase. Chromatography of this hypophasic layer yielded several pigmented and fluorescent spots, none of which corresponded to flavohygrocybin. In view of the observed degradation caused by hydrolysis and the failure to isolate an aglycone soluble in non-polar solvents, these investigations were not continued. If flavohygrocybin were a reduced sugar derivative as in riboflavin, hydrolysis as outlined above would not yield an aglycone.

For further analysis, an attempt was made to obtain a reasonably pure sample of flavohygrocybin. After removal of the amino compounds having the same R_f as flavohygrocybin by passage of the crude extract through a cation exchange column (p. 50-54), band chromatograms were prepared. Elution of the band corresponding to flavohygrocybin yielded

only a single spot upon subsequent chromatography and no other spots appeared under either short or long-wave ultraviolet illumination. Charring a similarly prepared thin layer chromatogram (TLC) also yielded only a single spot.

Attempts to produce flavohygrocybin in crystalline form were not successful. The dried residue of purified flavohygrocybin was an amorphous solid, dark brownish-yellow in color. Flavohygrocybin proved to be extremely hygroscopic and upon a short exposure to air the pigment changed to a gummy residue with the same color as the solid. This compound was very soluble in water, only sparingly soluble in methanol or ethanol and insoluble in the longer chain alcohols, acetone, or the non-polar organic solvents such as benzene or petroleum ether.

Visible and ultraviolet spectroscopy of flavohygrocybin showed but a single absorption maximum this at 420 nm (Figure 19), with an increase in absorption noted below 220 nm. A most unusual feature of this compound is the lack of absorption maxima between 220 and 400 nm.

In all cases, testing purified flavohygrocybin as well as the crude extracts with both sulfuric acid and Carr-Price reagent (p. 58) gave negative results indicating that these were not polyene pigments. Addition of sodium dithionite ($Na_2S_2O_4$) does not cause decolorization with the appearance of an absorption peak at 340 nm which indicates that this pigment is not a flavin (Robinson, 1967).

A sample of flavohygrocybin subjected to elemental analysis[5] yielded:

Carbon	:	40.10 % by weight
Hydrogen	:	6.89 % by weight
Nitrogen	:	24.70 % by weight
Oxygen	:	28.30 % by weight

This gives almost exactly, C_2H_4NO as an empirical formula.

Early attempts to obtain infrared spectra from flavohygrocybin were not successful. The difficulty was finally traced to the hygroscopic nature of this compound and successful spectra were obtained only when the pigment was kept under a vacuum before preparation. Potassium bromide pelletization was accomplished under dry nitrogen and the pellet was then returned to the vacuum chamber along with a dessicant and stored overnight before being examined. The results of two spectra are shown in Figures 20 and 21. These spectra represent two extremes observed from spectra of flavohygrocybin and differ notably in the peak observed at 1745 cm^{-1} (Figure 21) which is not well defined in Figure 20. Intergradations between these were also observed, and these differences did not appear to correlate with differing species. Rather, the differences represent some change occurring within the sample, as there was some variability in time from initial collection and preparation to the obtaining of spectra (six hours to several days, although care was taken to prevent oxidative change with all samples).

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[5]This analysis was accomplished through the courtesy of the University of Massachusetts Microanalysis Laboratory.

The strong absorption at 3380 cm^{-1} is indicative of either a hydroxy or carboxyl group (Bellamy, 1958; Jensen and Jensen, 1965). This suggestion that this pigment is hydroxylated is further strengthened by its high solubility in water and its extremely hygroscopic nature. The broad peak centered at 1035 cm^{-1} as well as the peak at 995 cm^{-1} could be attributed to C-O stretching and O-H deformation vibrations but more information on the actual structures to which the hydroxyl groups are attached would need to be known before final assignment of these bands could be made.

The presence of a carboxyl group is suggested additionally by the strong band at 1400 cm^{-1}, which again could be attributed to C-O stretching vibrations or O-H deformation.

Salt formation causes the characteristic carbonyl absorption to vanish (Bellamy, 1958) which may be the cause of the anomaly noted between the spectra shown in Figures 20 and 21. Ionization of a carboxyl group would cause the peak at 1745 cm^{-1} to be lost with a replacement band appearing between 1610 cm^{-1} and 1550 cm^{-1}. Inspection of Figure 20 shows that indeed no band at 1745 cm^{-1} is present, while if the band shape of the absorption at 1630 cm^{-1} is compared with Figure 21, the shape of this band in the latter Figure indicates perhaps that this band is masking another near 1600-1610 cm^{-1}. This aspect is not observed in Figure 21.

The IR spectrum shown in Figure 21 shows some similarity to the IR spectrum for Vulgaxanthin-I reported by Piatelli et al. (1965). The bands at 3380 cm^{-1}, 2930 cm^{-1}, 1745 cm^{-1}, 1630 cm^{-1}, and 1100 cm^{-1}

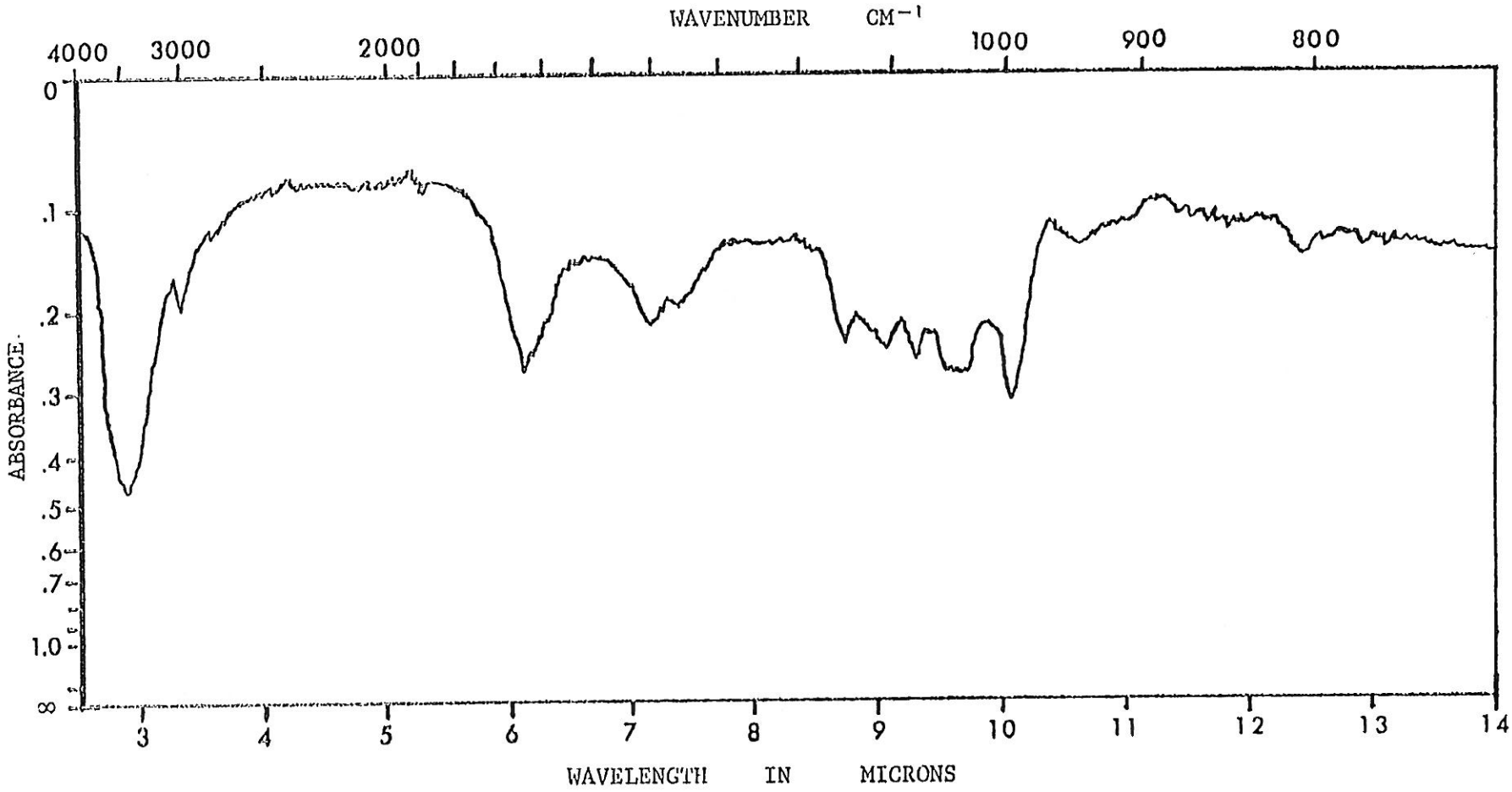

Figure 20. Infrared spectrum of flavohygrocybin from H. purpureofolius (#263). KBr pellet prepared under anhydrous nitrogen.

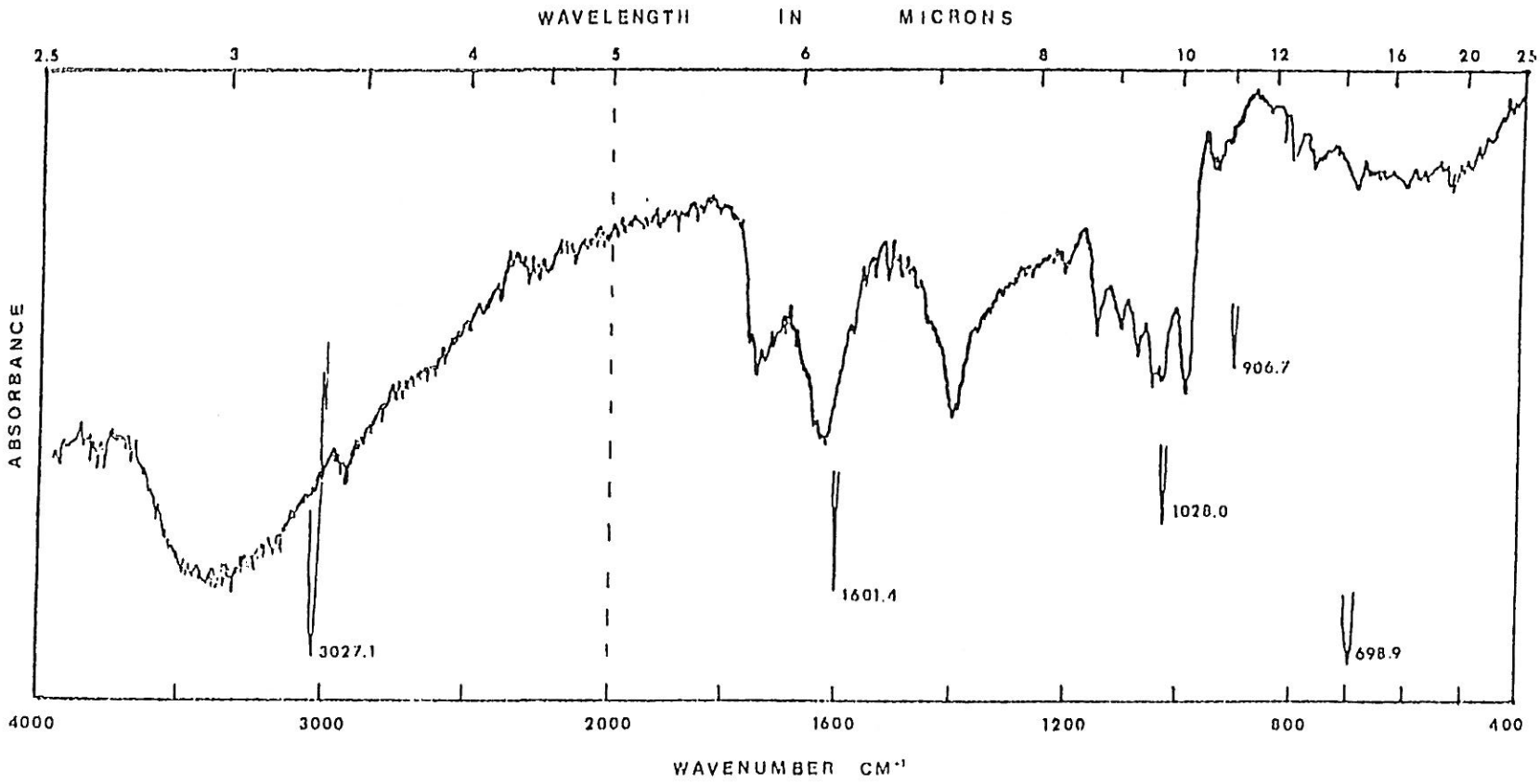

Figure 21. Infrared spectrum of flavohygrocybin from *H. flavescens*. Polystyrene used as reference.

correlate exactly. The possibility of a pyridine moiety being present in flavohygrocybin is strengthened by the presence of bands at 1630 cm^{-1}, 1150 cm^{-1}, 1075 cm^{-1}, and the weak absorptions at 800 cm^{-1} and 695 cm^{-1} (Bellamy, 1958).

NMR spectroscopy with a sample of flavohygrocybin dissolved in heavy water is shown in Figure 22. This result is difficult to interpret since there is no indication that the pigment has either aromatic or aliphatic properties (Paudler, 1971). However, this NMR spectrum is similar to that reported by Frauenfelder (1970) for russularhodin in $HFSO_3$. This may give weight to a consideration that flavohygrocybin is also a nitrogen-containing heterocyclic compound like russularhodin.

Since some of the analytical steps discussed above are more difficult to perform when water is used as a solvent, acetylation of the flavohygrocybin was attempted as detailed by Cason and Rapoport (1962). Although an acetylation product was obtained which was soluble in non-polar solvents, the chromophore was lost in the process. The nature of these colorless products and their relationship to the parent compound was not known, so further work was confined to the directly extractable pigment. Materials were limited due to spasmodic occurrence of sporophores.

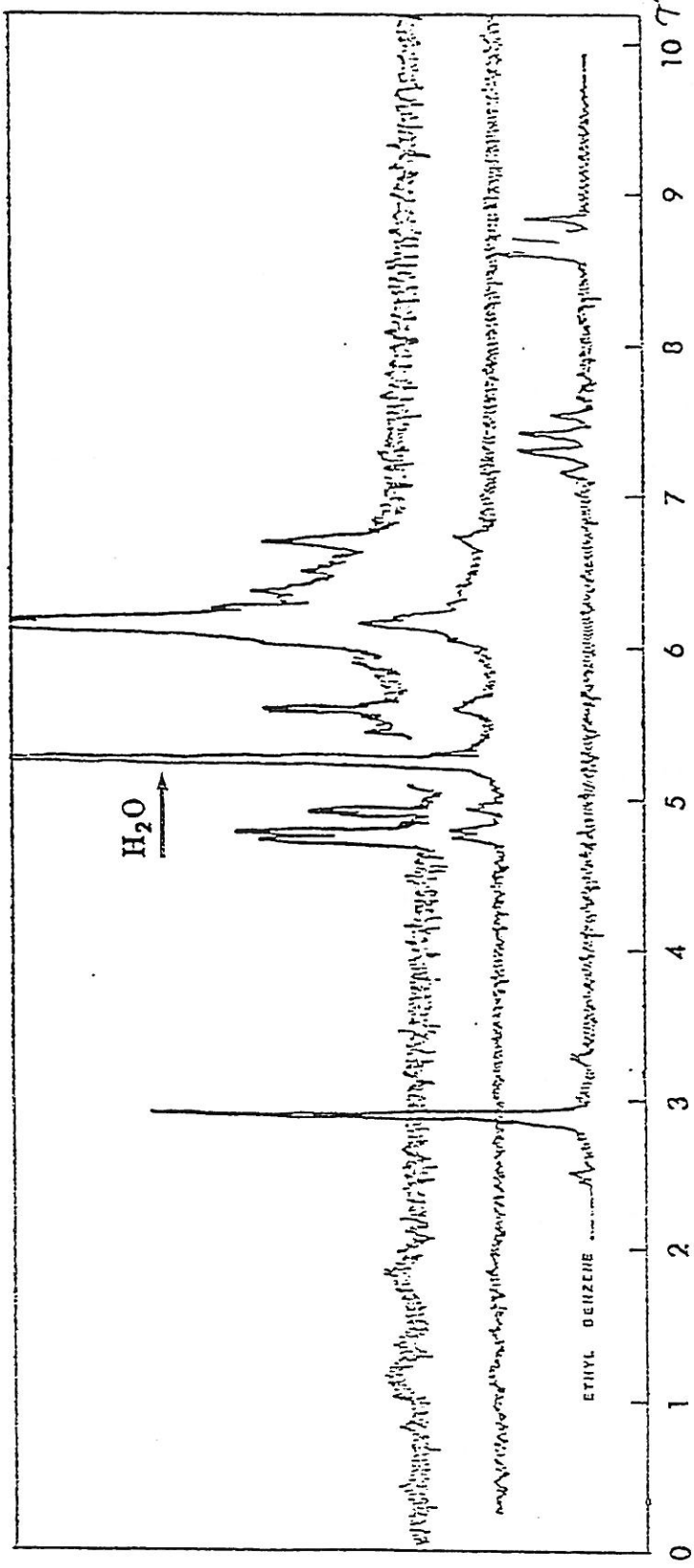

Figure 22. NMR spectrum of flavohygrocybin with ethylbenzene as reference.

CHAPTER IV

DISCUSSION

Color has been a character of major importance in defining taxa since the inception of study on agarics (Hesler and Smith, 1963; Singer, 1962; Fayod, 1889; Fries, 1938 and 1821), but the fundamental considerations on coloration and pigmentation were really first outlined by Arpin and Fiasson (1971):

1. The same color can result from entirely different chemical structures. Instead of demonstrating a relationship, a divergence would be indicated as the chemical structures involved do not have the same origin, for example:

a. Clitocybe venustissima (Fries) Saccardo and Hygrophopsis aurantiaca (Wulfen ex Fries) R. Maire, are both orange and this color could indicate an a priori support for grouping these together. However, the coloration in the first is carotenidic while that of the second is entirely different though of unknown composition (Arpin, 1966).

b. Both Hygrophorus cantharellus and a number of species of Cantharellus (cibarius, tubaeformis, cinnabarinus) have yellow to orange colorations, but the latter possess carotenes (Haxo, 1950; Fiasson and Arpin, 1967; Fiasson, 1968) as their coloring matter while the former contains flavohygrocybin and rhodohygrocybin which are quite distinct chemically from the carotenoids.

c. Russula lutea is yellow and owes its color to non-carotenidic

pigments (in most likelihood a pteridin derivative, Eugster and Frauenfelder, 1970), while the yellow Cantharellus species are quite different since these fungi contain carotenoids.

2. The same pigment in differing concentrations, may give differences in the color perceived. Phillipsia carminea (Patouillard) Le Gal is a bright red while Cookeina sulcipes (Berkeley) Kuntze is pinkish-fawn in color. Arpin and Liaaen-Jensen (1967a, b) note that the pigments have a very similar structure and the difference in perceived color is due only to a much greater concentration in the former species.

3. Two species which differ widely in color can still be closely related chemically. If the colors exhibited by polyene type pigments are examined, there is a range from colorless phytoene where the absorption is in the ultraviolet, to reddish-orange lycopene where the light absorbed is at much longer wavelengths (Weedon, 1965). Despite this difference, these compounds are biochemically quite similar. An analogous situation exists in Russula where colorless, yellow or red derivatives of pteridine have been found (Eugster and Frauenfelder, 1970).

4. The presence of two pigments whose chromophores are two of the subtractive colors (magenta, cyan, or yellow) can yield a color which could also result from a single pigment with a chromophoric group which produces one of the primary (red, blue, or green) colors. For example, the presence of two pigments, one magenta and the other yellow, would produce a red color. It is this color mixture which

has been found to be responsible for the brilliant red of some Hygrocybes. Red could also be produced by a single pigment, e.g. a polyene with a high degree of conjugation, or an anthraquinone derivative such as emodine.

Green can be produced from a mixture of two pigments, one with a chromophore resulting in a cyan color and the second with a chromophore which produces yellow. Apparently this is the situation in Leotia lubrica and in certain others in the Leotiaceae. Arpin (1968) found that certain cyan colored hydrosoluble pigments were present with some yellowish-orange lipophilic and carotenoid pigments. When these pigments are present simultaneously in a species of Leotia, they evoke the sensation of green.

With the preceeding data as background, the experiments and observations on Hygrocybes and other species were interpreted.

Identification of Flavohygrocybin and Rhodohygrocybin

Many of the basidiomycete pigments discussed earlier can be eliminated as being identical with flavohygrocybin simply on the basis of electronic absorption spectra. Most of the pigments have one or more absorption bands in the ultraviolet between 210 and 400 nm while none are found for flavohygrocybin. For this reason, flavohygrocybin is not a flavonoid as suggested by Sulya (1971). Flavonoids have two absorption bands--one between 310-560 nm which is the band responsible for their color, and a second in the ultraviolet between 240-290 nm.

Anthraquinones, which are soluble in aqueous solutions under special conditions, and the polyhydroxyanthraquinones are characterized by four absorption bands, three of which are in the ultraviolet. Anthranols have similar ultraviolet absorptions (Morton and Earlam, 1941; Spruitt, 1949; Briggs et al., 1952; Peter and Sumner, 1953; Birkenshaw, 1955; and Gabriel, 1959). The Borntträger reaction (Robinson, 1967) can test for anthraquinones and can also be used to differentiate between anthraquinones and naphthaquinones. Flavohygrocybin gave a negative Borntträger reaction, and on this basis as well as the other criteria listed above, flavohygrocybin is not an anthraquinone or naphthaquinone.

Of all the agarics other than the Hygrophorii which were examined, only Amanita muscaria, A. caesaria, and A. flavescens pigments showed similar solubilities and gave good chromatographic separations using the solvent systems which best separated the Hygrocybe pigments. It is known that these Amanita pigments are betalains (Eugster, 1973; Döpp and Musso, 1973a and b; Structure XL), while a seven membered heterocyclic is given as the basic component of the structure for these pigments (Von Ardenne et al., 1974; Structure XLIV). This suggests, along with the other data (IR, NMR, UV/VIS spectra, solubility characteristics and chromatographic separation) that flavohygrocybin may be of similar chemical structure. However, the empirical formula (C_2H_4NO) of flavohygrocybin is inconsistent with muscaflavin. Probably the analytical sample of flavohygrocybin is not free of all contaminants, although the amino

acid components were removed. This has now been confirmed independently by Besl et al. (1975). Ten species of Hygrophorus, viz. H. calyptraeformis, H. psitticinus, H. coccineus, H. miniatus, H. puniceus, H. conicus, H. acutoconicus, H. marginatus, H. hypothejus, and H. speciosus, examined by these authors were also included in this study. These authors and Von Ardenne et al. (1974) isolated a pigment which seems quite similar to flavohygrocybin from the above species except H. calyptraeformis, H. psitticinus, and H. marginatus. The pigment they studied differs from flavohygrocybin by having absorption maxima H^+, λ max=394 nm and OH^-, λ max=411 nm while flavohygrocybin has an absorption maximum at 420 nm. Besl's compound, muscaflavin, is an aldehyde whereas flavohygrocybin appears to be a muscaflavin derivative in which the oxygen of the aldehyde group is replaced by a nitrogen-containing moiety. This may account for the differences in the UV spectra. For nine species their results parallel this study, however flavohygrocybin was not isolated from H. speciosus.

Although rhodohygrocybin was found in much less quantity (see below) than flavohygrocybin, the limited available spectral data, solubilities and spot tests (pg. 140) indicate a strong possibility that this magenta pigment is also betalain. This would not be unexpected, as both the magenta betacyanins and yellow betaxanthins are found together in other plant genera where these pigment types are known to exist.

Unlike flavohygrocybin, rhodohygrocybin is nearly insoluble in

ethanol but freely soluble in water. As with flavohygrocybin, rhodohygrocybin is not soluble in the longer chain alcohols, acetone, or the nonpolar organic solvents.

When separated from flavohygrocybin by means of an ion exchange column (pg. 51) and then further purified by means of band paper chromatography, rhodohygrocybin still is strongly ninhydrin positive. Either all contaminant amino compounds had not been removed, or rhodohygrocybin unlike flavohygrocybin, has an amino acid group as an integral part of its structure. This is known to be the situation with the magenta colored betacyanin pigments, while some of the yellow betaxanthin pigments do not have an attached amino acid group (Mabry, 1966; Döpp and Musso, 1973). The small amount of purified rhodohygrocybin obtained appeared as a deeply magenta colored amorphous solid which was hygroscopic. Electronic spectra showed a maximum absorption in the visible, at 541 nm as shown in Figure 19. Additionally, a weaker absorption band was observed in the ultraviolet at 303 nm, with a minimum again at 285 nm, and then a gradual increase in absorption as 200 nm is approached.

The infrared spectrum of rhodohygrocybin isolated from Hygrophorus purpureofolius showed strong peaks at 3380 cm^{-1}, 1630 cm^{-1}, 1370 cm^{-1}, and 1100 cm^{-1}. Weaker absorption bands were noted at 2930 cm^{-1}, 1290 cm^{-1}, and 1230 cm^{-1}. There is a similarity of this spectrum with that of flavohygrocybin, but a difference in not having the fine structure noted for flavohygrocybin between 1150 cm^{-1} and 995 cm^{-1}. Rather this is replaced with a single

broad band with a maximum centered at 1100 cm^{-1}. Although this isolation of rhodohygrocybin was from H. purpureofolius, an agaric which provided the largest amounts of this pigment, the quality of this spectrum was not as good as that of flavohygrocybin. The intensities of infrared absorption were less than optimum due to an insufficient amount of rhodohygrocybin available for potassium bromide pelletization.

The data above suggest that rhodohygrocybin may also be a betalain type pigment. Earlier, when the violet colored betalain pigments of the Centrospermae (e.g. Beta vulgaris var. rubra, Portulaca grandiflora, Mirabilis jalapa) were being investigated by various workers, it became more evident as data accumulated that these pigments differed in many respects from other plant pigments. Dreiding (1961) summarized a series of tests which differentiated the then known betacyanins from other known plant pigments, particularly the anthocyanins with which the betacyanins had earlier thought to be similar. Tests with rhodohygrocybin isolated from H. miniatus and betacyanin isolated from red beets (Beta vulgaris cult. "Detroit Red") as well as Dreiding's notes on the reactions of the anthocyanin plant pigments are presented in Table 36. These tests suggest that rhodohygrocybin is a betacyanin type pigment, but as found with other fungal pigments of this type, the heterocyclic ring is a seven atom system corresponding to muscaflavin (XLIV) rather than the six atom system of betalamic acid (XLI).

Crude methanolic extracts yielded solids upon evaporation which were 1.21-1.55% of the whole weight of the sporophores before extraction. The amount of flavohygrocybin isolated from all species studied was fairly constant. This pigment accounted for 0.09-0.13% of the weight of the sporophores while rhodohygrocybin represented only 0.0065% in the brilliantly red colored Hygrocybes. Although the presence of rhodohygrocybin could be shown to be present in some collections of H. flavescens by chromatography, the percentage was less than 0.001% and could not be accurately determined. Spectrophotometric analysis demonstrates that flavohygrocybin and rhodohygrocybin account for approximately 80% of the visible light absorption by all pigments present in these agarics. The remaining 20% result from the minor pigments discussed below.

Other Pigments Found with Flavohygrocybin and Rhodohygrocybin

As seen in the tables and in figures 14 and 15, other pigments were found in much smaller quantities in addition to flavohygrocybin and rhodohygrocybin already discussed. Since the amounts isolated were insufficient for analysis, no analytical studies were made of these compounds other than noting their presence. The lack of one of these pigments in a species cannot be given much taxonomic significance, because the appearance, or lack thereof, of many of these pigments appear to be dependent upon the concentration of the crude extract applied to the chromatogram. Since variable quantities of

TABLE 36
REACTIONS OF RHODOHYGROCYBIN TO SIMPLE COLOR TESTS WHICH DIFFERENTIATE BETACYANINS FROM OTHER PLANT PIGMENTS. (ADAPTED FROM DREIDING, 1961)

Treatment	Pigment		
	Betacyanin	Rhodohygrocybin (from H. miniatus)	Anthocyanins
Addition of Alkali, KOH, NaOH	Rapid discoloration to yellow	Rapid discoloration to yellow	Slow discoloration to yellow via blue and green
Reacidification of alkaline solutions after some minutes	Red color cannot be regenerated.	Red color cannot be regenerated.	Red color reappears.
Addition of NH_4OH	Color remains for 30 sec. (R.T.)	Color remains for 20 sec. (R.T.)	As above for addition of alkali
Addition of mineral acids	Sol. becomes a darker violet, color destroyed in a hot acid solution.	Cold, color remains the same. Color destroyed in hot acid solution.	Sol. becomes lighter red. Precip. from conc. acid sol. on heating; precip. soluble in alcohol.
Addition of lead acetate	Reddish ppt.	Reddish ppt.	Blue-green or blue-gray ppt.
Addition of HNO_3	Immediate destruction of red color	Immediate destruction of red color	Only slow destruction of red color
Distribution between amyl alcohol & H_2O	Red color does not enter amyl alcohol at any pH.	Red color does not enter amyl alcohol at any pH.	Red color enters amyl alcohol at low pH.
Extraction from dried plant mtl. a) with ETOH b) with H_2O	a) no color b) all color	a) no color b) all color	a) some red color b) some red, becomes strongly red only upon acidification

the differing collections studied were obtained, the total amount of crude extracts applied to the chromatograms was often variable. For example, with the collection of H. parvulus studied, only two sporophores of this very small agaric were available for extractive purposes. Very few of the less abundant pigments seen in other Hygrocybes were found for H. parvulus (compare H. miniatus f. longipes, pg. 68). If more material were available for extraction, in most likelihood, these other bands might have been evident and useful. Elution of these minor pigments and testing with sulfuric acid or antimony trichloride in chloroform did give a negative test for polyenes.

However, an interesting observation was made concerning one of the unknown yellow pigments found between rhodohygrocybin and flavohygrocybin (R_f = 0.20-.23 acetone/water 7:3, R_f = 0.25-.33 acetone/water 6:4, R_f = 0.55-0.57 isopropanol/water 1:1). When young vigorously developing sporophores were used for extraction, and when this extract is used immediately to prepare chromatograms, this band appears gray. Under longwave ultraviolet this band is dark and nonfluorescent. Using older sporophores or a crude extract that has stood for several hours, this band is yellow and fluoresces under ultraviolet illumination. With the collection of Hygrophorus sp. #485, the yellow band (R_f = 0.24 acetone/water 6:4) is particularly intense.

Hygrophorus Pigments and Perceived Color

The relative amounts of rhodohygrocybin and flavohygrocybin seem to be important in determining the color of these fungi. In most of the species studied where flavohygrocybin was found, it was fairly constant in amount when compared to the total weight of the collection. Rhodohygrocybin was much more variable. In collections of H. flavescens where rhodohygrocybin was absent, the sporophores were a clear yellow in color. Those collections of this same agaric which were more orange in color had some rhodohygrocybin present. Other Hygrocybes which were brilliant red in color contained still larger amounts of rhodohygrocybin (these differences can be seen well in Figure 14. Compare the developed chromatogram for spot #4, H. flavescens, with spots #1 and #3, H. puniceus and H. coccineus, respectively).

The principle involved in the perceived color of these deeply red colored Hygrophori is that of a subtractive color process, similar in principle to the manner in which colors are produced by a reversal color transparency film such as Kodachrome or Ektachrome (Neblette, 1962). Flavohygrocybin, in appreciable concentration as found in H. flavescens, yellow form, absorbs most of the visible incident radiation of wavelength shorter than 480-500 nm. That is to say, no blue light is reflected. The only portions of the visible spectrum consist of wavelengths longer than 500 nm, that is, the spectral band between 500-700 nm. The sum total of the reflected

light in this spectral band will elicit the color "yellow" in an observer who has normal color discrimination (Judd and Wyszecki, 1963).

In appreciable concentration, rhodohygrocybin will strongly absorb radiant energy between 480-590 nm. Taken alone, rhodohygrocybin will allow two different spectral bands to be received by the observer: one band, from 400-480 nm, and a second from 600-700 nm. The first spectral band will elicit the color response "blue" while the second consists of energies which give the visual response of "red." When these two spectral bands are received in tandem by the observer as is the case with rhodohygrocybin alone, the visual response is "magenta" or "purple."

When both flavohygrocybin and rhodohygrocybin are present one of the two spectral bands transmitted by rhodohygrocybin is absorbed by flavohygrocybin. This is the band between 400-500 nm. Now the only spectral band perceived by the viewer will be the band between 600-700 nm. This band alone, will cause the visual perception of "red." This then, is the physical basis for the red color one sees in such agarics as Hygrophorus miniatus. Larger amounts of rhodohygrocybin cause the color "red" to be seen, while rhodohygrocybin in lesser amounts is observed as "orange." The yellow form of H. flavescens has no rhodohygrocybin, while the orange form has only a small amount (much less than that found in H. puniceus or H. coccineus c.f. figure 14, or H. miniatus).

As has been noted earlier, rhodohygrocybin is much more labile

than flavohygrocybin. This is the factor responsible for the fading to orange or orange-yellow observed in old sporophores of red Hygrophori such as H. miniatus or H. coccineus. As the chromophore of rhodohygrocybin is altered, the effect of flavohygrocybin becomes dominant as well as the degradation product of rhodohygrocybin which has a weak absorption at 486 nm. This accounts for a shift in color from red to orange or yellow in these older sporophores.

In very young sporophores there is a different situation. From the collection of H. puniceus (Figure 4) discussed earlier, there is the suggestion that sporophores collected before being exposed to any appreciable amount of light may be white. If collections of H. miniatus or H. strangulatus are sorted carefully so that chromatograms of very young sporophores are prepared separately, there is very little rhodohygrocybin. Only flavohygrocybin is present in large amounts (note the lighter, more yellow coloration in the young sporophore of H. strangulatus shown in Figure 7).

This developmental aspect of pigmentation may explain some of the anomolous color forms found within some species. H. nigrescens (Hesler and Smith, 1963) is distinguished from H. conicus as the former is said to be white when young. It is suggested here that H. nigrescens might be only a very young H. conicus which had been well covered with leaves. The pigment development had not yet been initiated, but it would proceed from colorless (no flavohygrocybin

exhibiting a positive Carr-Price test, exhibited the typical trimodal ultraviolet curve of a polyene (Scott, 1964; Weedon, 1965; Davies, 1965; Weedon, 1969; c.f. also, Figures 8, 10, and 11). Also, the spectral shifts expected in solvents of differing polarity (Weedon, 1965) are observed. These spectral data are summarized in Table 37.

For Mycena leaiana additional data are available. Results of IR spectroscopy is given in Table 29 and mass spectrometry yielded a molecular weight of 280 for this pigment.

These data indicate that this pigment is a polyene, but not a carotenoid as the molecular weight does not allow for the presence of isoprene-derived methyl groups on the conjugated skeleton of the pigment which would be typical of a carotenoid. If one does not consider the effects of other possible substituents, the absorption maxima would allow for this to be a straight chain polyene with nine conjugated double bands. As seen in Table 37, this pigment is very slightly soluble in petroleum ether and yields an absorption maximum of 414 nm for the central maximum. Scott (1964) gives 412.5 nm as the absorption maximum of the central peak for a straight chain polyene which has nine conjugated double bonds.

Infrared spectra of extracts from Mycena leaiana (Table 30) gave absorption maxima at 892 and 1218 cm^{-1}. The maximum at 892 cm^{-1} is consistent with the $(-C = C-)_n$ absorption of a polyene while the maximum at 1218 cm^{-1} quite possibly indicates the presence of a carboxyl group (-COOH). The occurrence of a carboxyl group

or rhodohygrocybin) to yellow (flavohygrocybin only) to red where both pigments are now present.

It is of interest to note that in collections of H. strangulatus where young sporophores were present, there appears a blue fluorescent band (acetone/water 7:3, R_f = 0.50-0.54; acetone/water 6:4, R_f 0.57) of higher R_f than flavohygrocybin. In addition, the chromatogram of the nearly colorless pileus of H. puniceus (p. 115) showed this band as well as a second blue fluorescent band (acetone/water 7:3, R_f = 0.73). This suggests that these colorless, blue fluorescing compounds found only in very young collections, may be precursors of flavohygrocybin.

Chromatography and Purification of Polyene Pigments

Several Hygrophori now classified in the section Hygrocybe (sensu Hesler and Smith, 1963) did not possess any of the pigments of the betalain type discussed above. Rather, the pigments present tested positive with the Carr-Price reagent and with sulfuric acid. The pigments present in H. marginatus var. concolor, H. marginatus var. marginatus, and H. psittacinus were soluble in non-polar solvents such as petroleum ether and were not soluble in water, while the pigments found in H. nitidus were soluble only in very polar solvents such as water. Similar in solubility to the pigments found in H. nitidus were pigments found in Mycena leaiana and M. epipterygia var. cespitosa. All of these pigments when purified in addition to

would suggest that this pigment may be acidic in nature (Bellamy, 1958, 1968). Scott (p. 79) gives some rules for correlating maxima and the length of the conjugated system for conjugated polyene acids. On this basis, $R(CH=CH)_7$-COOH would have a maximum of 418 nm. If R is a C_3 chain bearing an OH, the resulting structure $HO-C_3H_6-(CH=CH)_7$-COOH would have a MW of 286 and two polar functional groups consistent with the polar properties of the compound.

Straight chain polyenes are known in fungi. Cortisalin and Corticrocin have already been discussed previously. Polyene antifungal antibiotics produced by actinomycetes of the genus Streptomyces are discussed in detail, by Oroshnik and Mebane (1963). These compounds are linear polyenes. More recently, a group of straight chain polyene fungus pigments have been described by Badar et al. (1973). These polyenes are orange colored with absorption maxima around 410 nm and have molecular weights ~ 270.

Other than the chromatographic separations and electronic spectral data already reported, no other data is available on the pigments found in these agarics which possess pigments which appear to be polyene in nature.

None of the agarics which contained polyene pigments contained flavohygrocybin or rhodohygrocybin. In this sense, these agarics appear to be quite distinct from those where these latter pigments are found. Only one, H. psittacinus, appeared to contain a water soluble, cyan colored pigment which was lost immediately upon

TABLE 37
SPECTRAL ABSORPTION OF
POLYENES FROM VARIOUS
AGARICS

	MeOH	H_2O	Pet. Ether	C_2H_5-C_2H_5
H. marginatus var. concolor & var. marginatus	437		420i, 441, 462i	418, 438, 462
H. nitidus	380, 397, 415	382i, 404, 424i		378, 396, 418
H. psitticinus	445		404i, 424, 442i	
M. leaiana		415	392i, 414, 440i	414
M. epipterygia cespatosa	374, 392, 410			
	Pyridine	Benzene	ETOH	$CHCl_3$
H. marginatus var. concolor var. marginatus		443, 463, 490		
H. nitidus	389, 411, 433			
H. psitticinus				- 454, 475i
M. leaiana			382i, 405	396i, 420, 442i
M. epipterygia var. cespatosa				

extraction. It is probable that this pigment is responsible for the evanescent green coloration of this agaric. The nature of this extremely fugaceous cyan pigment in H. psittacinus is not known.

It must be noted that with the polyene pigments considered here, there may be two distinctly different types present. Evidence has been given to show that the polyene in M. leaiana is not a carotenoid. Such cannot definitely be said about the other species. In particular, the pigments in both varieties of H. marginatus and in H. psitticinus, which are soluble in non-polar solvents may be carotenoids. Additional data are needed to prove or disprove this suggestion. However this may be, there is still a distinct hiatus between the Hygrophori which contain flavohygrocybin and rhodohygrocybin as compared to those Hygrophori which contain polyene type pigments.

Taxonomic Implications

The idea of using biochemical data to supplement morphological and ecological characters to derive a better system of classification of organisms is not a new one. For example, Savile (1955) suggested that biochemistry along with cellular morphology may be more reliable indicators of affinities than gross morphology in lower plants where simple structure makes comparative morphology difficult. With few available morphological characters, differing taxonomic views can

develop in taxa and indeed this has happened in the genus Hygrophorus (Orton and Watling, 1969).

Other workers have used biochemical data along with the more classical taxonomic criteria. Gabriel used information on pigments with some species of Cortinarius, and more recently, Bresinsky (1974) used the chemical analysis of pigments to substantiate an infrageneric relationship in Suillus. He showed that the section Larigni (sensu Singer, 1962) is characterized by grevillin-B. Another pigment, different from but believed to be related to grevillin-B, is found in the section Suillus. Additionally, Bresinsky studied the pigments produced in mycelial cultures of Suillus and found them to be different from, but related biogenetically to the pigments found in the sporophores. Citing studies of Gaylord et al. (1970), Gaylord and Brady (1971), as well as his own investigations, Bresinsky proposed that the Paxillaceae, Gomphidiaceae, Rhizopogonaceae, and perhaps the Coniophoraceae are related to the Boletaceae. Twenty-one of twenty-nine species belonging to these families were found to produce typical pulvinic acids in culture (of the eight remaining species, five species would not grow in culture).

From chemical differences between pigments found in some anascosporus yeasts, Fiasson (1972) was able to clarify the taxonomic and phylogenetic position of the species studied. He found that species such as Candida marina Van Uden et Zobell and Bullera alba (Han) Derx contained carotenoids while Sporobolomyces puniceus

Komata and Nakase were quite different in pigment composition.

Thus, there are precedents for using the analytical chemical information of pigments to elucidate the taxonomic and phylogenetic positions of organisms within the Eumycota.

With reference to species within the genus Hygrophorus, Hesler and Smith (1963) observed:

> "Here again we have a series of taxa distinguished on progressive differences of pigmentation, a situation occurring throughout the genus as a whole and one which, it is hoped, can some day be studied from the standpoint of the chemistry involved."

The data in Table 38 summarize the chemical and the morphological/anatomical information on the species of Hygrophorus examined during this study. Within this table, there is a group of species in which there is a positive correlation between flavohygrocybin, parallel gill trama, possession of clamps, and a lack of pigments which test positive with either sulfuric acid or antimony trichloride. Within this group of species, rhodohygrocybin may be either present or absent, but wherever this pigment is found, flavohygrocybin is also found. Historically, all of these species have been considered to form a closely-knit natural group since the time of Fries (1821, 1838). This close relationship is now substantiated by the additional character of similarity of pigmentation. The common denominator found in this group of species which is centered about H. miniatus is the presence of flavohygrocybin.

Rhodohygrocybin is more variable in occurrence and appears to be more important as a character at the species level. This aspect

is shown best with H. parvulus where rhodohygrocybin is found in the stipes, while absent from the pileus. In H. flavescens rhodohygrocybin varies between collections, but is always found in smaller amounts than in species such as H. miniatus. This magenta pigment is unusually abundant in H. purpureofolius and accounts for the unusual purplish gill color in this species. In addition to H. purpureofolius, the only other species in which rhodohygrocybin is found in greater quantity than in H. miniatus, is Hygrophorus sp. #485. The latter is also unusual in having a large quantity of an unknown yellow pigment of R_f = 0.24 (acetone/water 6:4) in addition to flavohygrocybin. Some other Hygrophori have traces of this yellow pigment, but not in the quantity found in Hygrophorus #485.

A yellowish-orange band, R_f = 0.04 (acetone/water 7:3) and R_f = 0.06 (acetone/water 6:4) is found in a few species (viz H. flavescens yellow form (W), H. conicus orange form (W), H. conicus red form (M), and H. acutoconicus) and its presence may indicate some relationship between some species of subsection Punicei to others placed in the series Conici of subsection Hygrocybe.

The property of blackening when bruised, is peculiar to H. conicus and some closely related species. Collections of both orange and red forms of H. conicus were studied and the crude extract of both was a black, nearly opaque solution. The sporophores turned completely black during the extraction procedure. The extract produced

TABLE 38

(Continued)

Species	Clamps	Gill Trama	Polyene Test	Flavo-hygrocybin	Rhodo-hygrocybin
H. #485	+	P	-	+++	++++
H. #489	+	P	-	+++	+++
H. hypothejus	+	DIV	-	+	-
H. speciosus	+	DIV	-	-	-
H. marginatus	-	I	+	-	-
H. psitticinus var. psitticinus	-	I	+	-	-
H. nitidus	INF	I	+	-	-
H. pratensis	+	I	-	-	-

TABLE 38

SPECIES OF *HYGROPHORUS* COMPARED WITH RESPECT TO SOME ANATOMICAL AND CHEMICAL CHARACTERS

+ possess clamps; - do not possess clamps; INF = clamps infrequent; P = parall. gill trama; SP = subparallel; I = interwoven; DIV = divergent. For flavohygrocybin and rhodohygrocybin, - not present, + present in small amount to +++ abundant.

Species	Clamps	Gill Trama	Polyene Test	Flavo-hygrocybin	Rhodo-hygrocybin
Hygrophorus flavescens	+	P	-	+++	+ or -
H. cantharellus	+	SP	-	+++	++
H. strangulatus	+	SP	-	+++	+++
H. miniatus var. *miniatus*	+	SP	-	+++	+++
H. miniatus f. *longipes*	+	P	-	+++	+++
H. parvulus	+	SP	-	pileus +++ stipe +++	- +
H. coccineus	+	SP	-	+++	+++
H. puniceus	+	SP	-	+++	+++
H. purpureofolius	+	P		++	++++
H. conicus	+	P	-	+++	++
H. acutoconicus	+	P	-	++	?
H. turundus var. *sphagnophilus*	+	SP	-	+++	+++
H. cuspidatus	+	P	-	+++	+++
H. subminutulus	+	SP	-	+++	+++

a black spot when spotted at the origin of the chromatogram. Upon development, however, two dark greyish spots (R_f = 0.00 and R_f = 0.91-0.94 acetone/water 7:3) were found, as well as the other brightly colored pigments and the latter were undiminished in saturation at their expected R_f values. The orange form was found to lack rhodohygrocybin, but two weak orange bands were found R_f = 0.04 and R_f = 0.07. Flavohygrocybin was found in both the orange and red forms and thus confirms the relationship of H. conicus to other species in the section.

No substantiation can be given to the argument to separate some of these species (e.g. H. conicus) into a separate genus (or section), Godfrinia as proposed by Maire (1902), or to this genus in expanded form by Herink (1958), since these species contain the same pigments as do other Hygrocybes. As noted by Lange (1935-1940) and by Smith and Hesler (1942), taxonomic importance cannot be given to the production of two-spored basidia as in some cases both two-, three- and four-spored basidia are found on the same sporophore.

All of the species of Hygrophorus discussed thus far have been considered to be closely related by most modern mycologists. However, opinions differ on whether this group of species represents a genus (Lange, 1923; Singer, 1962; Moser, 1950, 1967; Orton and Watling, 1969) a subgenus (Bataille, 1910; Nuesch, 1922; Smith and Hesler, 1942; Kühner and Rogmanesi, 1953; Smith and Hesler, 1954), or a section (Orton, 1960; Hesler and Smith, 1963). The

results of the present analyses seem to support the thesis that these species are related together in a group by genetic mechanisms in common which are responsible for the evolution of pigments.

A number of modern agaricologists recognize Hygrocybe as a genus rather than as a section. Again, recognizing this group to be natural, Orton and Watling (1969) note some disagreement among mycologists as to the selection of the type species for Hygrocybe. These authors follow Donk (1949) and Singer (1962) and select Hygrocybe conica (Fries) Kumner as the type species for Hygrocybe. Singer in 1959 and Hesler and Smith (1963) selected Hygrophorus miniatus (Fries) Fries. The results of this study indicate that the basic concept of Hygrocybe is not changed whether H. miniatus or H. conica is considered as the type since both contain flavohygrocybin and rhodohygrocybin and have the additional generic characteristics enumerated below.

Integrating the results of this study into the concept of Hygrocybe, the species have the following characters: hymenophoral trama which is parallel to slightly interwoven; basidia which are four- or two-spored; clamp connections present; a lamellar attachment which varies from adnexed to decurrent; spores which are white in deposit, smooth and inamyloid; stipes which are longitudinally innately striate or glassy smooth, which may be either dry or glutinous, are always non-annulate, and additionally are hollow at maturity; latex not present; sporophore color often quite bright, ranging from vivid red through yellow, flavohygrocybin is always

present, rhodohygrocybin often present but variable in amount.

Several controversies about the position of certain species of Hygrophorus appear to be resolved by the information derived from pigment analysis. Kaufmann in an unpublished manuscript (Hesler and Smith, 1963) placed H. cantharellus in section Camarophyllus while retaining H. miniatus in Hygrocybe. Hesler and Smith believed these species to be too closely related to warrant placement in different sections. Pigment analysis substantiates their view as flavohygrocybin and rhodohygrocybin are found in both H. cantharellus and H. miniatus.

Since H. pratensis (Fries) Fries has been shown not to possess flavohygrocybin, this study does not substantiate the view of Orton and Watling (1969) that Hygrophorus pratensis should be placed in Hygrocybe. The pigment of H. pratensis could not be removed from the sporophore when water, acetone, methanol or petroleum ether were used as extractive solvents. Both water and methanol should have removed the pigments if they were the same as in H. flavescens. If the pigments were carotenoids, methanol, acetone, or petroleum ether should have been effective for removal. The pigments responsible for the coloration of H. pratensis do not resemble any of the others which were found in this study. A different pigment composition and an intricately interwoven gill trama are evidence for the exclusion of H. pratensis from Hygrocybe.

The pigment separations observed with Hygrophorus hypothejus (section Hygrophorus, Hesler and Smith, 1963) presented a somewhat

different aspect. This was not unexpected, as this species has long been considered separate from the Hygrocybes because of the divergent gill trama of H. hypothejus. The sporophores after methanolic extraction retained an amber color in the pileus which could not be removed with any other solvents. The clear yellow extract yielded a major yellow pigment, R_f = 0.20 (acetone/water 6:4). Flavohygrocybin was present in very small quantity, less than in the other species studied, suggesting some relationship with Hygrocybe. Additionally, four colorless but blue fluorescing bands, R_f = 0.08, 0.18, 0.32 and 0.98 (acetone/water 6:4) were observed which are unique to this species. Another colorless band R_f = 0.77 (acetone/water 6:4) may correlate with the blue fluorescing pigment found in some Hygrocybes (p. 85).

Hygrophorus speciosus var. speciosus also placed in section Hygrophorus, gave a good separation of pigments when chromatograms were prepared and developed in solvents routinely used for the separation of rhodohygrocybin and flavohygrocybin. However, nine yellow bands were present which did not correlate with any of those previously determined in Hygrocybe and there was an intense orange band at R_f = 0.30. Solubility characteristics, chromatographic separation and ultraviolet fluorescence, suggest that these pigments may be related to those found in Hygrocybe. The absorption spectrum of a major yellow pigment, R_f = 0.42 (acetone/water 6:4) which was partially purified by chromatography, is very similar in appearance to that obtained from flavohygrocybin, but the absorption

maximum in water, is at 488 nm, differing from that observed for flavohygrocybin in H. hypothejus.

My results are similar to those obtained by Besl et al. (1975) for H. hypothejus and H. speciosus but in the latter their results were uncertain due to scanty amounts. The existence of pigments which appear to be chemically related to flavohygrocybin, as well as the traces of this pigment in H. hypothejus, may indicate a possible phylogenetic relationship between Hygrocybe and the subsection Hygrophorus of section Hygrophorus. A taxonomic hiatus should be maintained though as H. speciosus and H. hypothejus differ by gelatinous veils, divergent gill trama and a proven mycorrhizal relationship with conifers (Trappe, 1962; Singer, 1962).

The presence of nonpolar polyene pigments in Hygrophorus marginatus and the lack of flavohygrocybin or any other water soluble pigment, indicates a division between this species and the Hygrocybes which contain flavohygrocybin. The latter tested negative for polyene type pigments. This biochemical dissimilarity supports the view held by Singer (1958) of a basic difference between H. marginatus and Hygrocybes such as H. flavescens and H. miniatus. He writes:

> "...I have observed chemical differences between Humidicutis here Hygrophorus marginatus sensu Smith and Hesler and Hygrocybe differences which I have demonstrated to the mycologists present in the 1953 season at Cheboygan, Michigan and which consisted in the following:
>
> KOH (10%) dissolves the pigment of 'Hygrophorus marginatus' and destroys it. In the same solution Hygrocybe flavescens and other true Hygrocybes available at that time did not show any such action of KOH and the pieces of carpophores

> left in KOH remained colored. Anilin (sic) stains the lamellae of 'Hygrophorus' marginatus sordid olive brown while on the lamellae of Hygrocybe flavescens, a characteristic orange-salmon discoloration was observed. The conclusion that the corresponding pigment in Hygrocybe is not identical with that of 'Hygrocybe' marginatus does not appear to be far fetched or unreasonable. This observation, on the contrary gives more weight to direct observation in the field suggesting that the Ridgway and Maerz and Paul values of the colors involved are different from those obtained Hygrocybes studied by me in this regard."

In the classification given by Hesler and Smith (1963) H. marginatus var. concolor and var. marginatus are placed in section Hygrocybe of Hygrophorus. Singer (1948, 1951) placed H. marginatus in Tricholoma, subgenus Humidicutis. Later, he (1958) moved this species to the Hygrophoraceae, genus Humidicutis. European authors (Orton, 1960); Orton and Watling (1969); Moser (1967); Kühner and Romagnesi (1953), do not consider H. marginatus in their classifications, as it apparently occurs only in North America.

There is a hiatus between H. marginatus and such Hygrocybes as H. conicus and H. flavescens. Examination of Table 38 shows that in addition to a major difference in pigment type, H. marginatus and its varieties lack clamps and have an interwoven hymenophoral trama. Additionally, this species lacks flavohygrocybin and rhodohygrocybin. This group of characters indicates that H. marginatus should be removed from Hygrocybe.

In view of the morphological and chemical similarities and differences which exist between H. marginatus and H. flavescens and

related species, I propose a reconciliation of the opinions of Hesler and Smith (1963) and Singer (1975) by recognizing Humidicutis as a section of Hygrophorus. There is no doubt that there are basic differences between Hygrocybe and Humidicutis, but whether these constitute the characters of subgenera, sections, or some other infrageneric taxa will be interpreted variously by investigators. As understood here, Humidicutis would embrace those species which lack rhodohygrocybin and/or flavohygrocybin, containing instead pigments which are polyene in nature. Since H. marginatus is the type of the section, one must associate with this agaric only those species which have essential characters in common with H. marginatus. Singer recognizes a probable difference in pigmentation of Humidicutis from Hygrocybe, a hymenophoral trama which is not bilateral (in fact, a trama which is somewhat interwoven as contrasted to the parallel trama of Hygrocybe), smooth spores, lamellae which are adnate or emarginate but not deeply decurrent, and the absence of clamp connections.

Perhaps H. psitticinus and H. nitidus should be placed in Humidicutis rather than Hygrocybe for these two species have polyene pigments and no flavohygrocybin or rhodohygrocybin. However, occasional clamp connections do occur in H. nitidus and H. psitticinus and including such species would diffuse the concept of Humidicutis. There are further problems if H. nitidus is placed in Humidicutis for the lamellae are decurrent and the polyenes have differences in polarity. The decurrent lamellae are probably not

a significant departure as other sections in Hygrophorus have variable attachment.

The polarity differences noted between the polyenes found in H. marginatus and H. nitidus would be suggestive of differences in structure. It is possible to have linear polyenes which would have some spectral and other chemical characteristics in common with carotenoid pigments which are isoprenic in structure. If there is a difference, it would be indicative of differing biochemical pathways of synthesis, and rather than indicating a close relationship would suggest some separation of these taxa.

It appears with the available evidence that there may be a relationship or coincidence between the frequency of clamps on hyphae of the basidiocarp of a species and the polarity of the polyene pigment. H. marginatus and related species have polyene pigments freely soluble in petroleum ether and lack clamps. In H. psitticinus clamps occur at a few septa of the hyphae of the epicutis but are not uncommon at the base of basidia. With H. nitidus which has the most polar polyene, clamps are frequent, being present throughout the pileus tissue. At this time, it is difficult to say with certainty that the above relationship holds; this decision must await the study of other species which would belong in this complex as well as the determination of the chemical structure of these pigments.

In "The Agaricales in Modern Taxonomy" (1975) Singer recognizes H. marginata (apparently both var. concolor and var. marginatus sensu

Hesler and Smith, 1963 as well as var. olivacea), and H. czuica as the species comprising Humidicutis.

According to the dominant wavelengths of recorded color, there may be other species now assigned to Hygrocybe (Singer, 1975) which have polyene pigments rather than the flavohygrocybin or rhodohygrocybin. For example, in section Minutulae Singer, H. flavifolia and H. ceracea have colors reported which seem characteristic of polyenes and thus a transfer to Humidicutis may be necessary. This conclusion results from a colorimetric analysis where the recorded colors were plotted on a C.I.E. graph. Since these species have not been examined by the author, actual assignments cannot be made at this time.

In conclusion, it appears that the Hygrophori which contain rhodohygrocybin and/or flavohygrocybin form a "close knit natural group" quite distinct from the Hygrophori which contain polyene pigments. This difference as well as other correlative characters are summarized in Table 38.

Phylogenetic Considerations

Relationship of Hygrophorus to Cantharellus.

The concept of a relationship between Hygrophorus and Cantharellus was expressed as early as 1838 by Fries. The first species listed under the "Tribe" Hygrocybe by Fries (H. cinnabarrinus sic) is considered today to be a Cantharellus. He notes under H. cantharellus, the second species described under this "tribe", "cum priori, cui affinis et colore simul Canth. cibarium referens." Fayod (1889) recognized Hygrocybe as a genus in what today would be considered a family (the Cantharellaceae). Grouping by Fayod yields Cantharellus, Camarophyllus, Hygrophorus, and Hygrocybe as genera. He also observed that Morgan considered Cantharellus cinnabarinus as a Hygrocybe, and states that Hygrocybe cantharellus has some relationship with the Chanterelles (Cantharellus). Thus it is seen that the concept of a phylogenetic relationship between Hygrophorus and Cantharellus developed quite early in the history of agaricology.

More recently, mycologists have had differing views concerning a phylogenetic link between Cantharellus and Hygrocybe. Singer (1975) discusses Cantharellus under his "Genera excludenda" believing that this genus is not at all related to the Agaricales but rather to the Clavariineae. Hesler and Smith however (1963) postulate a phylogenetic relationship of Hygrocybe with Cantharellus where the latter represents the more primitive condition.

The direction of fungal evolution in the Hymenomycetes appears to be in the direction of maximal efficiency of spore dispersal and this involves an increase in the spore-bearing area without wastefully increasing the volume of the sporophore. It is on this basis as well as other characters, that agarics like Hygrophorus have been considered by some mycologists as a natural progression from Cantharellus since only adding lamellae would increase spore production.

There are multiple reasons for considering that Hygrophorus (particularly Hygrocybe ss. lat.) has evolved from the Cantharellaceae (and in particular, Cantharellus) as suggested by Hesler and Smith (1963). There is a general resemblance in stature, color, gymnocarpic mode of development, in some a lack of cystidia on the hymenium, long basidia, a waxen appearance of the hymenophore, spores which are light colored and are similar in shape, the presence of clamps on hyphae of the sporophore, and sporophore production in similar habitats on similar substrata. In both genera, the possession of gymnocarpy, elongate basidia, and a hymenium, which at best is composed of thick lamellae which are rather widely spaced, present a group of characters which many mycologists now consider to be representative of a primitive condition in agaricalian development. With species of Hygrocybe, a distinctly lamellate hymenophore with an organized trama is found, and in some species, simple cystidia also occur; these characters are considered more advanced than the trama, lamellar configuration and cystidia found

in the Cantharellaceae.

In addition to these differences in structures, an abrupt change from stichobasidia as found in Cantharellus to chiastobasidia in Hygrophorus is observed. These changes are all consistent with evolutionary advancement from Cantharellus to Hygrophorus (Hesler and Smith, 1963). These authors suggest that the waxy hymenophore and elongate basidia found in both genera are the two major characters which indicate a relationship between these genera.

Cantharellus and Craterellus produce pileate, gymnocarpic agaric-like sporophores which are fleshy, putrescent, and which do not perennate or revive after dessication. The pileus is developed by marginal growth from the stem apex and the hymenium varies from nearly smooth (Craterellus) to being irregularly lamellate. Often, the sporophores are aromatic, the odor reminding one of dried apricots. The lamellate-appearing hymenophore is formed by obtuse gill golds with fertile edges which often dichotomize and anastomose. The basidia are elongate, narrow and stichic, and elongate, curved sterigmata are found on the basidia. Clamp connections are present on the hyphae of Cantharellus but not on those of Craterellus. The spores in either genus vary from white to yellowish or pinkish. (In contrast, the spores of Hygrocybe are chalk white.)

The yellow to orange colors in the basidiocarps of Cantharellus are caused by the presence of carotenoids (Fiasson, 1968). The dark colored fungi (e.g. Craterellus cornucopioides) also produce carotenoids as well as dark pigments of another type which are not

easily removed from the sporophore with the usual solvents. As a result of carotenogenesis as well as the possession of stichobasidia, Arpin and Fiasson (1972) believe that Craterellus and Cantharellus should be combined.

The species of Hygrocybe centered about H. miniatus have never been shown to possess carotenoids. In this study the Hygrocybes which most often have been considered as related to Cantharellus (e.g. H. cantharellus) possess rhodohygrocybin and flavohygrocybin, pigments most unlike the carotenoids in their chemical behavior and structure.

The development of the obtuse ridges in Cantharellus is the result of linear areas of excessive intercalary growth of the initially smooth hymenium. These obtuse ridges radiate from the stem apex towards the pileus margin in the direction of hyphal growth. In this development of gills, there is not the outgrowth of gill hyphae to form a new tramal plate to support the new hymenium, but instead there is only increased intercalation of new basidia from the subhymenium. This results in a gill-fold which possesses an obtuse fertile edge from the outset and lacks a parallel trama. The trama that does exist represents hyphae which originally were from the lower portion of the pileus. These hyphae become pulled out in various directions as the basidial connections of these hyphae are separated in the expanding hymenium. This development may result in simply a loose weft of hyphae in the trama or a microscopic gap where the gill-fold joins with the pileus.

Additionally, there is a continual outgrowth of new basidia which overtop the old basidia. If these older basidia do not dissolve away, they may be detected as being imbedded in the hymenium. The process continues, and the gill-fold deepens by continued intercalary growth and thickening by basidial outgrowth (Corner, 1950). I have observed this phenomenon with C. cibarius along the Gulf Coast. When the weather is continuously wet, these fungi will persist for a month or more, continually shedding spores. Microscopic examination discloses that it is the overtopping of new basidia which permits continual spore formation and dissemination. It is entirely possible that the relative longevity of these fungi coupled with the continual production of basidia under optimum conditions, may represent an alternative answer to a hymenophore of increased surface area. Interestingly, under these same conditions of heat and humidity, species of Hygrocybe have a lifetime of only a few days. In true agarics, including Hygrocybe, the gill is formed by a definite outgrowth of hyphae more or less at right angles to the hyphae in the pileus; the gill edge is sterile, at least while in active growth. The hymenium is precisely constructed from a single layer of basidia, with the new being inserted between the old without overtopping. The gill thickness in Hygrophorus is principally caused by the descending tramal hyphae.

In summary, there are differences between Hygrocybe and Cantharellus. These are:

a) Differences in pigments. Cantharellus (sensu stricto) has

carotenoids in all species studied, while the pigments found in Hygrocybe as shown here and very recently also noted by von Ardenne et al. (1974) and Besl et al. (1975) are very different chemically.

b) Major differences in the developmental aspects of the hymenium of the two genera.

c) Differences in odor. Cantharellus is often highly aromatic while Hygrocybe is odorless or somewhat unpleasant in odor.

d) The presence of overtopping basidia in Cantharellus, thus allowing for continual spore dissemination over a long period of time. Basidia of Hygrophorus do not overtop.

e) The presence of pale but nevertheless definitely colored spores in Cantharellus, this color due to the presence of carotenoids. Hygrocybe always has white spores.

These characters as a group do not enhance the possibility of a relationship between section Hygrocybe and Cantharellus. I believe that the similarities between the two genera are superficial and an example of convergence.

Although Hygrocybe has been considered by some investigators to be advanced with respect to Cantharellus, this section possesses many characters which are considered to be primitive: elongate basidia, undifferentiated cystidia, gymnocarpic development. It

appears possible that the basidiocarps of Hygrocybe may be more primitive and represent the archetype for the evolution of some other agaricalian genera.

This study, as well as those of von Ardenne et al. (1974) and Besl et al. (1975), has shown that some species of Amanita possess some pigments which appear to be identical with some of those found in Hygrocybe. Furthermore, this study has shown that a small amount of flavohygrocybin is present in H. hypothejus. This species of the subgenus Hygrophorus has been considered to represent an evolutionary advancement with respect to Hygrocybe by Hesler and Smith (1963). H. hypothejus has a divergent gill trama similar to that found in Amanita. This species also has a glutinous outer veil which produces a viscid pileus and a stipe whose lower portion is also viscid from the gelatinization of superficial hyphae. Might not these characters suggest an evolutionary development in the direction of Amanita? The answer to this intriguing question must await further biochemical and anatomical studies.

The possibility of a relationship in Hygrophorus to Cantharellus is increased if the section Humidicutis is considered. This study has demonstrated the presence of polyene pigments in species of this section, and particularly interesting are the nonpolar polyenes found in H. marginatus and H. psitticinus. However, it must be proven that these polyenes are carotenoids before the relationship is convincing.

Relationship of Hygrocybe to Mycena.

Both Singer (1962) and Hesler and Smith (1963) suggest that there is a possible phylogenetic relationship between Hygrophorus and Mycena.

Mycena, a genus of white-spored, rather small agarics, is one of the genera comprising the Tricholomataceae. Mycena is characterized as having a pileus which may be variously shaped, but is usually conic or convex, a margin which is usually straight and appressed against the stipe when young, but sometimes incurved at first; sporophores that are fragile, fleshy, or somewhat cartilaginous in consistency but hardly reviving when remoistened, and usually membranous to submembranous. The stipe is cartilaginous, tubular or hollow, glabrous or appressed-fibrillose, while the lamellae are usually ascending-adnate or hooked, often arcuate and occasionally decurrent. The spores are white in deposit, thin-walled, smooth, amyloid reaction either positive or negative. Cystidia are usually present and the markings, shape and distribution of the cystidia are among the most important characters used to distinguish species of Mycena.

The North American species of Mycena were treated comprehensively by Smith (1947) and the infrageneric divisions of interest in this discussion are given as follows:

1. Subgenus Glutinipes has species where the stipe in cross-section show an outer gelatinous layer. As a result, the stipe is viscid to the touch when fresh. The following

sections are of concern here:

a) Section Caespitosae has species which are conspicuously cespitose and lignicolous, and have viscid pilei. Also of interest, all the species of this section are found only in North America.

b) Section Viscosae, comprising species with viscid pilei which are humus-dwelling and have stipes with greenish or greenish-gray tints.

2. Subgenus Eumycena is the largest of the subgenera and contains species with smooth spores, lacks a basal disc or bulb on the stipe and has a nongelatinous stipe. Eumycena is partitioned into ten sections two of which are of interest here:

a) Section Adonidae has white or brightly colored species whose gills are not marginate.

b) Section Calodontes contains species which are brightly colored or mixed with fuscous.

Hesler and Smith (1963) note that in a search of the Agaricales for relationships with Hygrophorus, the section Hygrocybe (sensu Hesler and Smith) seems to hold the best possibilities, especially through the section Adonidae of subgenus Eumycena of Mycena. Fayod (1889) was the first to suggest a relationship between Hygrocybe and Mycena, but he suggested that the brightly colored Calodontes probably have affinities with Hygrocybe.

In the course of this study, only two representatives of the Adonidae were collected--Mycena amabilissima (Peck) Saccardo and Mycena pura (Fr) Quélet. The pigments from M. amabilissima were not extractable in either polar or nonpolar solvents. In this respect, the pigments were not similar to either the pigments of H. miniatus or the polyenes isolated from H. marginatus or H. nitidus. Similarly, the pigments of M. pura did not resemble those of Hygrocybe. These results are not encouraging to demonstrate a relationship between Hygrophorus and the section Adonidae of Mycena. The color changes described for M. adonis (Fries) S. F. Gray of this same section are very suggestive of the color changes noted for some Hygrocybes and it would be interesting to study this Mycena. Such color changes are not noted for M. amabilissima and M. pura.

Unfortunately, no representatives of the section Calodontes were found during the period of the investigation, and there are no data on pigments of this group in the literature.

Two representatives of subgenus Glutinipes were studied, Mycena leaiana of section Caespitosae, and M. epipterygia var. caespitosa of Viscosae. The pigments found in both species proved to be very polar polyenes and thus very similar to the pigments found in H. nitidus. The unusual feature of these pigments is their extreme solubility in water and their near insolubility in the type of solvents in which most carotenoids are soluble. The molecular weight of 280 for the polyene in M. leaiana also indicates that this pigment

is not a carotenoid. The isoprenic nature of carotenoids yields molecular weights close to 500.

Since polyenes were found in section Humidicutis of Hygrophorus, a comparison was indicated between other characters of M. leaiana, M. epipterygia var. caespitosa and those of H. marginatus of Humidicutis.

There are a number of serious differences:

(1) H. marginatus lacks clamp connections of hyphae of the basidiocarp while these are present in the two Mycenas.

(2) The spores are inamyloid in H. marginatus but amyloid in M. leaiana and M. epipterygia var. caespitosa.

(3) The Mycenas of concern have dextrinoid or weakly amyloid tissues in the pileus.

(4) Gelatinous tissues are well-developed in the two species of Mycena but absent in H. marginatus.

(5) H. marginatus is terrestrial while both Mycenas are lignicolous.

(6) H. marginatus lacks differentiated cystidia on the lamellae, while they are present in the Mycenas.

(7) The differences in basidial length and waxy hymenophore appear to be minor, but certainly cannot be considered positive.

(8) H. marginatus has nonpolar polyenes, while those of M. leaiana and M. epipterygia var. caespitosa are very polar.

Each of these differences taken singly, or even in limited combinations, could be viewed as only the differences between species, but there are no strong positive unusual characters to indicate a close relationship between H. marginatus and the two Mycenas. The common presence of polyene pigments may indeed show a connection, but it is certainly an obscure one in view of the many differences. Perhaps the analysis of pigments in more species will clarify the relationship.

The studies of Fiasson, Arpin and Gabriel were reviewed in the introduction. Their investigations indicated that the understanding of the chemical nature and differences in structure of agaric pigments were most useful in uncovering relationships which, on the basis of anatomical features, were not definitive. The production of a pigment (e.g. a carotenoid), often involves many metabolic steps. For this reason, the absence of a pigment or of its precursors is indicative of a major difference in the genome. For example, in this study, both rhodohygrocybin and flavohygrocybin appear to be complex nitrogen containing heterocyclic compounds probably related to the betalains. The metabolic pathways to produce these pigments would be quite different from those for the production of carotenoids such as found in Cantharellus. From this aspect then, the major structural differences in pigments confirm for multiple differences in the genomes of the two groups being compared. From the functional point of view, the more universal a function is, the more widespread are the compounds

which are required for the execution of that function (e.g. glucose and ATP, elements in metabolic chains essential to life). The more restrictive a function, the more specific are the compounds associated with that function. Apparently, agaric pigments are in this latter class (Fiasson, 1968). For these reasons, agaric pigmentation appears to be uniquely suited for studies of agaric taxonomy and phylogeny.

SUMMARY

1. Nineteen species of Hygrophorus subgenus Hygrocybe sensu Hesler and Smith, were examined for pigment composition by paper chromatography and the determination of electronic, IR, and NMR spectroscopy. In one group of species centered about H. miniatus, a group of water soluble pigments were more abundant than the others and are the pigments primarily responsible for their coloration. Since these pigments appeared to be undescribed in the literature, they were named "rhodohygrocybin" and "flavohygrocybin." However, some similar pigments were found in three species of Amanita.
2. UV, visible, IR, and NMR spectroscopic data are given for flavohygrocybin. Preliminary evidence indicates that this compound may be a nitrogen containing heterocyclic compound, perhaps similar to the betalains, but no structure assignment is made at this time. Evidence is presented that rhodohygrocybin probably is also related to this group of compounds.
3. It is shown that the variable color observed in some Hygrocybes is due to variations in the ratios of rhodohygrocybin and flavohygrocybin. Furthermore, the destruction of rhodohygrocybin leaving flavohygrocybin in the red Hygrocybes accounts for the fading to yellow commonly observed in these agarics.
4. Another group of pigments in other species of Hygrocybe, later assigned to the section Humidicutis, is demonstrated to be

polyene in nature. These polyenes differ in polarity. The polyenes isolated from H. marginatus are soluble in non-polar solvents while the polyenes isolated from H. nitidus and Mycena leaiana are freely soluble in water. This latter pigment is shown by mass spectroscopy to have a molecular weight of 280.

5. A relatively simple test to screen agarics for the presence of polyenes is described and discussed.
6. A modification of section Hygrocybe of Hygrophorus is proposed. Hygrocybe is restricted to those Hygrophori which have rhodohygrocybin and/or flavohygrocybin, while Humidicutis contains only those Hygrophori which have polyene pigments.
7. The problem of a phylogenetic relationship between Cantharellus and Hygrophorus is discussed.
8. A possible phylogenetic relationship between section Humidicutis of Hygrophorus and the subgenus Glutinipes of Mycena is discussed.

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APPENDIX A - TABLE 39

CAROTENOIDS IN SELECTED BASIDIOMYCETES

APPENDIX A - TABLE 39

CAROTENOIDS IN SELECTED BASIDIOMYCETES

Species	Carotenoid		Reference
Clavariaceae			
Clavaria cardinalis Boud. & Pat.	carotenoids present		Heim (1949)
C. helicoides Pat. var. *robusta* Corner	β-carotene dehydro 3,4 torulene dehydro 2'plectaxanthin hydroxy β-carotene		Fiasson (1968)
C. pallida, B. & C.	carotenoids not found		Fiasson (1968)
C. corniculata Fries	"	"	
C. aurea Fries	"	"	
C. formosa Fries	"	"	
C. fistulosa Fries	"	"	
C. truncata Quél.	"	"	
C. pistillaris Fries	"	"	
Pistillaria micans Fries	β & γ carotene torularhodin ester of torularhodin		Fiasson (1968)
Clavulinopsis fusiformis (Fries) Corner	none		Fiasson et al. (1969)
Cl. aurantio-cinnabarina (Schw.) Corner var. *aurantio-cinnabarina*	Neurosporene γ carotene β-carotene	+ 5% 84.5%	Fiasson et al. (1969)

TABLE 39, CONT'D

Species	Carotenoid	Reference
Cl. aurantio-cinnabarina (Schw.) Corner var. amoena (Zoll et. Mohr) Pat.	γ carotene 1.5% β-carotene 95 %	Fiasson et al. (1969)
Cantharellaceae		
Cantharellus cinnabarinus Schw.	canthaxanthin phytofluene β-carotene echinenone	Haxo (1950)
C. cibarius Fries	lycopene β-carotene	Willstaedt (1937); Fiasson (1968)
C. cibarius Fries var. amethystinus Quél.	none	Fiasson (1968)
C. tubaeformis (Fries) Quél.	phytofluene 1.4% cis neurosporene 2 % β-carotene 1.6% neurosporene 72 % lycopene 8.8% di-hydroxy β-carotene 13.2% others	Turian (1960); Fiasson and Arpin (1967); Willstaedt (1937); Fiasson (1968)
C. cibarius Fries var. pallidifolius Smith	lycopene trace P 426 7 % P 444 5.2% γ carotene 7 % β-carotene 67 % others 13.7%	Fiasson et al. (1970)

TABLE 39, CONT'D

Species	Carotenoid		Reference
Cantharellaceae Cont'd			
C. minor Peck	P 444	22.7%	Fiasson et al. (1969)
	β-carotene	57.6%	
	others	19.7%	
C. infundibiliformis Fries	neurosporene	74 %	Fiasson et al. (1969)
	lycopene	8.8%	
	others	17.2%	
C. friesii Quélet	neurosporene	0.7%	Fiasson (1968); Fiasson et al. (1969); Arpin et al. (1968)
	γ carotene	1.7%	
	β-carotene	43.9%	
	echinenone	32 %	
	canthaxanthin	9.1%	
C. lutescens Fries	neurosporene	4.5%	Fiasson & Arpin (1967); Turian (1960); Willstaedt (1937); Fiasson (1968)
	lycopene		
C. konradii (R. Maire) Kühner & Romagnesi	colorless compounds only λ max 282, 292, 303 & 319 nm		Fiasson (1968)
Craterellus cornucopioides Fries	neurosporene	60 %	Fiasson (1968); Fiasson et al. (1969)
	lycopene	40 %	
Cr. fallax Smith	lycopene	18.7%	Fiasson et al. (1969)
	γ carotene	21.3%	
	β-carotene	49.5%	

TABLE 39, CONT'D

Species	Carotenoid		Reference
Cantharellaceae Cont'd			
Cr. odoratus (Schw.) Fries	lycopene	+	Fiasson et al. (1969)
	P 426	1.4%	
	γ carotene	12.3%	
	β-carotene	60.2%	
	others	3.5%	
Cr. cinereus (Fries) Quel.	none		Fiasson et al. (1969)
Gomphus floccosus (Schw.) Singer	none		Fiasson et al. (1969)
Agaricales			
Clitocybe venustissima (Fries) Saccardo	β-carotene	8 %	Arpin (1966); Fiasson (1968)
	carotene	75 %	
	others	17 %	
Omphalia chrysophylla (Fries) Kummer	β-carotene	87 %	Fiasson (1968)
	γ carotene	11 %	
	lycopene	0.5%	
	torulene	1.5%	
O. abiegna (Berk. & Br.) J. Lange	none		Fiasson (1968)
Phyllotopsis nidulans (Fries) Gilbert & Donk	β-carotene	58 %	Fiasson (1968)
	γ carotene	29 %	
	neurosporene	0.5%	
	echinenone	8 %	
	others	4.5%	

TABLE 39, CONT'D

Species	Carotenoid	Reference
Agaricales Cont'd		
Lactarius torminosus Fries		
a) mycelium	β-carotene	Jayko et al. (1962)
b) sporophore	none	Fiasson (1968)

APPENDIX B

LIST OF SPECIES COLLECTED

Given below is a listing of the collections examined during the course of this study. The collection numbers are Cibula collection numbers and the collections are on deposit in the University of Massachusetts Mycological Herbarium, (MASS), Amherst, MA. In some cases, insufficient material was available for both extraction and herbarium preservation. These collections are prefixed with the date of collection.

Abbreviations used for locations are:

HEF = Harrison Experimental Forest, De Soto National Forest, Saucier, MS.

NSTL = National Space Technology Laboratories, Bay, St. Louis, MS.

UMBS = University of Michigan Biological Station, Pellston, MI.

Amanita caesarea

435 Gravel Pits, NSTL, 6 September 1973.

514 Noxubee Wildlife Refuge, MS, 17 September 1974.

A. flavoconia

9 August 1972, So. Deerfield, MA.

A. muscaria

10 September 1970, Mt. Toby St. Forest, MA.

405 HEF, Saucier, MS, 7 December 1972.

406 (Deep scarlet) HEF, MS, 7 December 1972.

409 HEF, MS, 7 December 1972.

27 November 1974, HEF, MS.

7 December 1974, Santa Rosa, MS.

Cantharellus cinnabarinus

90 So. River Rec. Area, Conway, MA, 29 July 1969.

109 Baptist Hill, Conway, MA, 10 August 1969.

8 August 1974, HEF, MS.

C. cibarius

75 Pine Hill Rd., Conway, MA, 1 July 1969.

91 So. River Rec. Area, Conway, MA, 29 July 1969.

195 Conway, MA, 6 August 1970.

Clavaria formosa

508 Tombigbee National Forest, Van Fleet, MS, 20 September 1974.

Clavaria pulchra

9 August 1972, So. Deerfield, MA.

Clitocybe aurantiaca

15 September 1968, Mt. Toby St. Forest, MA.

Cortinarius sanguineus

442 NSTL, 8 September 1973.

C. semisanguineus

470 HEF, 31 January 1974.

Craterellus cantharellus

14 July 1968, Shaker Lakes, Cleveland Hts., Ohio.

C. cornucopioides

339 So. Deerfield, MA, 8 August 1972.

Gomphus floccosus

333 Conway St. Forest, MA, 26 July 1972.

341 Roaring Brook Rd., Conway, MA, 6 August 1972.

342 Ashfield, MA, 13 August 1972.

Hygrophorus #485

485 HEF, 8 August 1974.

554 HEF, 16 June 1975.

Hygrophorus #489

489 HEF, 20 August 1974.

555 HEF, 16 June 1975.

556 HEF, 16 June 1975.

H. acutoconicus

21 August 1967, UMBS.

H. borealis

7 October 1970, So. Deerfield, MA.

H. calyptraeformis

September 1973, Cedarville St. Pk, MD, Collected by J. Ammirati.

H. cantharellus

74 Pine Hill Rd., Conway, MA, 1 July 1969.

89 So. River Rec. Area, Conway, MA, 29 July 1969.

98 Pelham, MA, 5 August 1969.

100 Mt. Toby St. Forest, MA, 5 August 1969.

116 Wendell St. Forest, MA, 12 August 1969.

264 Savoy Mountain St. Forest, MA, 18 August 1971.

358 N. Leverett, MA, 19 August 1972.

H. coccineus

235 Mt. Toby St. Forest, MA, 7 October 1970.

H. conicus

73 Rt. 202, New Salem, MA, 18 June 1969.

111 (Red form), Baptist Hill, Conway, MA, 10 August 1969.

112 (Orange form), Baptist Hill, Conway, MA, 10 August 1969.

252 Albany, NY, 28 October 1970.

367 Orono, ME, 22 September 1972.

394 Smith's Falls, Ont., 7 October 1972.

H. cuspidatus

5 August 1971, Audubon Nature Ctr., Sharon, CT.

H. flavodiscus

239 Mt. Toby St. Forest, MA, 28 October 1970.

H. flavescens

99 Mt. Toby St. Forest, MA, 5 August 1969.

107 DAR St. Forest, Goshen, MA, 7 August 1969.

199 Mt. Toby St. Forest, MA, 12 September 1970 (Yellow form)

200 Mt. Toby St. Forest, MA, 12 September 1970 (Orange form).

214 " " " " " 20 September 1970.

230 " " " " " 28 September 1970.

238 " " " " " 7 October 1970.

254 Audubon Nature Ctr., Sharon, CT, August 1971.

329 Lee Rd., So. Deerfield, MA, 21 July 1971.

330 " " " " " 26 July 1971.

352 N. Leverett, MA, 19 August 1972.

H. hypothejus

528 Picayune, MS, 22 December 1974.

24 December 1974, Picayune, MS.

H. marginatus var. concolor

70 N. Leverett, MA, 17 July 1969.

103 DAR St. Forest, Goshen, MA, 7 August 1969.

163 Tahquamenon Falls St. Park, MI, 12 September 1969.

217 N. Leverett, MA, 19 September 1970.

353 " " " 19 August 1972.

354 Savoy Mtn. St. Forest, MA, 21 August 1972.

H. marginatus var. marginatus

71 N. Leverett, MA, 17 July 1969.

368 Orono, ME, 22 September 1972.

H. miniatus f. longipes

220 N. Leverett, MA, 19 September 1970.

331 Conway St. Forest, MA, 26 July 1972.

382 Orono, ME, 23 September 1972.

H. miniatus var. miniatus

97 Pelham, MA, 5 August 1969.

105 DAR St. Forest, Goshen, MA, 7 August 1969.

117 Wendell St. Forest, MA, 12 August 1969.

119 Leverett, MA, 14 August 1969.

121 " " 22 August 1969.

340 Pine Hill Rd., Conway, MA, 6 August 1972.

361 Savoy Mtn. St. Forest, MA, 21 August 1972.

372 Orono, ME, 23 September 1972.

492 HEF, MS, 22 August 1974.

H. nitidus

69 N. Leverett, MA, 17 July 1969.

106 DAR St. Forest, Goshen, MA, 7 August 1969.

122 N. Leverett, MA, 21 August 1969.

206 " " " 11 September 1970.

218 " " " 19 September 1970.

357 " " " 19 August 1972.

H. parvulus

101 Conway, MA, 5 August 1969.

6 September 1970, Leverett, MA.

H. pratensis

231 Mt. Toby St. Forest, MA, 7 October 1970.

H. psitticinus

113 Baptist Hill, Conway, MA, 11 August 1969.

120 Whatley Rd., Conway, MA, 14 August 1969.

136 Cheboygan, MI, 9 September 1969.

250 Albany, NY, 28 October 1970, Collected by R. Habermehl.

390 Gower, Ontario, Canada, 6 October 1972.

H. puniceus

118 Whatley Rd., Conway, MA, 14 August 1969.

215 Mt. Toby St. Forest, MA, 20 September 1970.

392 Le Gateau Provincial Pk., Quebec, Canada, 7 October 1972.

H. purpureofolius

263 Savoy Mtn. St. Forest, Florida, MA, 18 August 1971. Type locality

H. speciosus var. speciosus

232 Cook St. Forest, MA, 8 October 1970.

396 " " " " 21 October 1972.

H. strangulatus

201 N. Leverett, MA, 11 September 1970.

216 Mt. Toby St. Forest, MA, 20 September 1970.

229 " " " " " 28 September 1970.

237 " " " " " 8 October 1970.

H. subminutulus

522 HEF, MS, 10 December 1974.

H. subviolaceous

371 Orono, ME, 23 September 1972.

H. turundus var. sphagnophilus

369 Orono, ME, 23 September 1972.

383 Hawley Bog, MA, 30 September 1972.

Hypomyces lactifluorum

79 N. Leverett, MA, 17 July 1969.

565 HEF, MS, 16 June 1975.

Lactarius thyinos

23 September 1972. Orono, ME.

Lepiota lutea

433 NSTL, MS, 31 August 1973.

Mycena amabilissima

391 Ramsayville (Ottawa), Ontario, Canada, 7 October 1972.

H. epipterygia var. cespatosa

295 Janice Landing, MS, 24 December 1971.

M. haematopus

6 September 1970, N. Leverett, MA.

207 N. Leverett, MA.

M. leaiana

362 Florida, MA, 21 August 1972.

28 June 1973, Pushaw Pond Rd., Penobscot Co., ME; collected by R. Homola.

M. pura

236 Mt. Toby St. Forest, MA, 7 October 1970.

393 Le Gateau Provincial Pk., Quebec, Canada, 7 October 1972.

Naematoloma fasciculare

9 December 1974, HEF, MS.

Polyporus cinnabarinus

22 September 1970, Colrain, MA.

P. sulphureus

26 September 1968, Conway, MA.

Rhodophyllus salmonellas

August 1967, Pellston, MI.

Russula lutea

28 August 1966, Chesterland, Ohio.

R. veternosa

21 July 1972, So. Deerfield, MA.

26 July 1972, Conway St. Forest, MA.

Suillus decipiens

481 HEF, MS, 6 February 1974.

Tricholoma flavovirens

370 Orono, ME, 23 September 1972.

533 HEF, MS, 23 December 1974.

538 HEF, MS, 30 December 1974.

APPENDIX C

Several species of Hygrophorus were encountered during this study which did not correspond to known species. Accordingly, a complete description of each is given here. Color notation used is that of Munsell (Munsell Color Company, 1967) and equivalents are given where possible, in terms of Ridgway color names (Ridgway, 1912).[6] Also given for each Munsell notation is the more general, descriptive color name in ISCC-NBS nomenclature (Kelly and Judd, 1955). The order of this nomenclature as it appears in the description is: Munsell, (Ridgway equivalent/ISCC-NBS color name).

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[6] These equivalents are based on both data of Rayner (1970) and unpublished calculations of the author.

Hygrophorus #485

PILEUS: 5-8(10) mm broad, convex to flattened, becoming broadly convex-depressed; color 6.25 R 3/12 (carmine/#11, vivid red) to 7.5 R 3/12 (sl. deeper than scarlet red/#11, vivid red); somewhat lighter nearer margin, 7.5 R 3.2/10 (no Ridgway equivalent/#13, deep red), with a very narrow ($\simeq$ 250μ) white to yellowish margin in some mature specimens which contrasts with the deep red disc, red color retained well in older basidiocarps; not viscid; surface composed of radially arranged, highly pigmented hyphae (Figure 13) which group together in upturned fasicles giving the pileus a scurfy to squamulose appearance, this aspect more pronounced near the disc where the squamules have a circumferential arrangement and increase in size toward the center. CONTEXT: thin, hygrophanous, reddish. LAMELLAE: convexly arcuate, subdistant, emarginate-decurrant, broad but acuminate on the stipe, thick, somewhat triangular; only one tier of lamellulae; color 7.5 YR 7.5/5 (close to light pinkish-cinnamon/between #73, pale orange-yellow and #76, light yellowish-brown) to 10 YR 9/4 (close to pale ochraceous-salmon/between #73, pale orange-yellow and #89, pale yellow), occasionally with a slight reddish hue, 10 R 5/8 (near vinaceous rufus/between #28, light yellowish-pink and #29, moderate yellowish-pink); edges even, but in some mature sporophores the edge is eroded. STIPE: 2.5-3 cm X 1.0 mm, over entire length somewhat lighter in color than pileus, 7.5 R 4/14 (close to Nopal red/#11, vivid red), darker near base, somewhat lighter near juncture with pileus, here 10 R 5/10 (sl. darker than Carnelian Red/#37,

moderate reddish-orange); cylindrical, not viscid, glabrous with a silken sheen; hollow; interior context fibrous, concolorous with exterior. SPORES: chalky white in deposit, inamyloid, hyaline in H_2O mount; ellipsoid to subovoid, 7.5-8.4 X 4.7-5.8μ; thin-walled with a distinct apiculus; contents in fresh collections are granular. BASIDIA: clavate, hyaline, 31-38 X 7-9.4μ; all appear four spored. GILL TRAMA: parallel, hyphae ≃ 12μ in width; hyaline in water mount; clamps observed. CONTEXT: hyphae irregularly subglobose, 25-40 X 25μ, in H_2O mount appear hyaline. CUTICULAR HYPHAE: strongly pigmented, thin-walled cells, 95-205 X 18.7μ (Figure 13), the pigment is evenly distributed through the cell. The hyphae immediately below the strongly pigmented surface hyphae are similar in size and shape, but when seen individually, appear hyaline. Seen en masse, these hyphae assume a yellowish color. PLEUROCYSTIDIA and CHEILOCYSTIDIA: not observed. CLAMPS: observed on hyphae of gill trama. HABIT, HABITAT and DISTRIBUTION: gregarious beneath mixed hardwood and pine, adjacent to Loblolly Pine, Block IV, Plot 4, Fertilization Study Plots, Harrison Experimental Forest, De Soto National Forest, Saucier, MS, on a raised mound of earth and stumps. One collection (#485), consisted of numerous sporophores. MATERIAL STUDIED: #485, De Soto National Forest, Saucier, MS, 8 August 1974;

agaric appears to be closely related to H. trinitensis var. firmus Dennis (Dennis, 1953; Hesler and Smith, 1963). Basidia lengths agree, while the spore sizes fall within the range for var. trinitensis. However, this collection does differ in the generally more cream colored gills. Dennis describes the gills in his Trinidad collection as being coral colored. There is also close similarity to Hygrocybe mexicana Singer (Singer, 1958) but this species is reported as having a glabrous pileus. Spore and basidia sizes agree. This hygrophorus differs from H. firmus Berkeley and Broome var. firmus (Berkeley and Broome, 1871; Singer, 1957; Hesler and Smith, 1963) in not having dimorphous basidia and spores. Furthermore, in their original publication, Berkeley and Broome report that the pilei of their collection (#880) was yellow and minutely tomentose. Although apparently related to H. cantharellus, this agaric is distinguished by its more diminutive stature, more saturated red coloration of the pileus and stipe, a probably more southern distribution, as well as the rather strong yellow pigment band at R_f = 0.24 (acetone/water 6:4) noted on chromatograms prepared from crude pigment extracts. Also, this species does not "fit" any of the seventeen Malayan varieties of H. firmus described by Corner (1936).

Hygrophorus #489

PILEUS: 1.3-4 cm broad; plano-convex to convex-depressed, becoming depressed; margin finally upturned and then infundibuliform; color very variable from deep red to buff with some caps showing shades of greenish-yellow, this variability due both to drying and to areas which were covered by debris--when moist, 5 R 3/4 (somewhat lighter than "maroon"/#16, dark red) to 7.5 R 2/4 (close to "maroon"/#44, dark reddish-brown) to 7.5 R 3/10 (between "garnet brown" and "carmine"/#13, deep red) to 10 R 3/6 ("Morocco red"/#43, moderate reddish-brown), some yellowish areas 2.5 Y 7/8 (close to "primuline" yellow/#87, moderate yellow) at extreme margin and where pileus is rimose; when dry, pileus then 2.5 YR 5/4 ("fawn color"/#42, light reddish-brown) to 2.5 YR 5/8 (close to "ferruginous"/#54, brownish-orange), one very dry pileus 5 Y 6/3.2 ("Dark olive buff"/#91, dark greyish-yellow); where covered by leaves, other debris or adjacent pilei, 2.5 Y 8/2 (no equivalent, closest to "pale olive buff"/#89, pale yellow) to 2.5 Y 6/2 (no equivalent, /#94, light olive green) to 5 Y 6/2 (between "light greyish-olive" and dark "olive buff"/#112, light olive-grey) to 2.5 Y 7/8 (close to "primuline yellow"/#87, moderate yellow) to 5 Y 5.4/6 (between "olive-lake" and "pyrite yellow"/#106, light olive); entire surface covered with abundant radially arranged hyphae which give a fibrillose to fibrillose-squamulose disc; not viscid; at times rimose; margin even, somewhat eroded to eroded-plicate in older sporophores. CONTEXT: thin;

hygrophanous; yellowish to reddish in color. LAMELLAE: broadly adnate to decurrent becoming decurrent in maturity; also quite variable in color both among differing basidiocarps and on a single specimen being cream colored 5 Y 9/3 (between "ivory yellow" and "cream colored"/#89, pale yellow) to 7.5 Y 9/3 (closest to "massicot yellow"/#104, pale greenish-yellow) while some exhibit reddish colors, 10 R 5/6 ("terra cotta"/#39, greyish-reddish-orange) while others exhibit greenish-yellow hues, 7.5 Y 7/2 (no Ridgway equivalent/#93, yellowish-grey) to 10 Y 7/4 (no Ridgway equivalent/ #105, greyish-greenish-yellow) to 10 Y 9/4 (sulfur yellow/#104, pale greenish-yellow) all being observed; three tiers of lamellulae; distant to subdistant; the edges, especially in older material, are noticeably eroded, appearing almost serrate. STIPE: 3-6.5 cm long, 5-9 mm broad, red, upper third, 7.5 R 2.5/9 (no Ridgway equivalent, closest to "garnet brown"/between #16, dark red and #13, deep red) and 10 R 5/10 (close to "carnelian red"/#37, moderate reddish-orange), paling both above and below, becoming yellow closer to the base, 5 YR 5/8 (no Ridgway equivalent; lighter than "amber brown"/#54, brownish-orange) to 5 Y 8.5/8 (close to "empire yellow"/#86, light yellow) while at the base, the color is nearly white; interior hygrophanous, 5 Y 8.5/6 ("baryta yellow"/#86, light yellow); terete, equal to somewhat enlarged near the apex; not viscid; glabrous with a silken sheen; hollow. SPORES: chalky white in deposit; some 12-17 X 7-10μ, others 6.5-8.5 X 4-5.5μ very variable in size; thin-walled; smooth; ellipsoid to ovate; hyaline in KOH; inamyloid. BASIDIA: hyaline; narrowly

clavate, 35-40 X 6-9.4μ. CHEILOCYSTIDIA: numerous, clavate with a umbonate apex; contents granular; thick-walled, 45-65 X 9.4-12.5μ. GILL TRAMA: parallel; 115-170 X 14-18.7μ; hyaline. CUTICLE: distinctly two-layered, the uppermost composed of non-pigmented radially arranged hyphae which form a trichodermium; the hyphae 58-150 X 11.5-21μ; this hyaline to very pale yellow layer is 55-115μ deep. Second layer of radially arranged and interwoven hyphae, strongly pigmented a deep red; 47-150 X 16-21μ. CLAMP CONNECTIONS: present on hyphae in gill trama. HABIT, HABITAT, and DISTRIBUTION: gregarious to subcespitose; found in low areas chiefly beneath sweet bay (Magnolia virginiana) in leaf litter, Mississippi Gulf Coast, summer. MATERIALS STUDIED: #489, 20 August 1974; #555 and #556, 16 June 1975; #567, 24 July 1975; #571 and #572, 10 August 1975; all collections Harrison Experimental Forest, De Soto National Forest, Saucier, MS. OBSERVATIONS: The larger spores (12-17 X 7-10μ) and the abundant cheilocystidia, suggest a relationship with H. appalachianensis but this Hygrophorus differs from this species and all others in the subgenus Hygrocybe examined in this study by both the hyaline trichodermium which overlay the pigmented hyphae of the cuticle, and by the extreme variability in spore size. The non-pigmented trichodermium is in part, responsible for the variable color observed in this agaric due to differing optical properties when wet or dry. When the sporophore is wet as for example, after a recent rain, water fills the interstices between these hyphae. Under these conditions, the differences in the index of refraction

between the water, cell wall and cell contents (N_1 = 1.33 vs $N_2 \simeq$ 1.3-1.5) are not great. Hence, surface reflections from these hyphae are minimized allowing most of the light to pass through this layer and then to undergo preferential absorption of selected wavelengths from the pigmented hyphae below. However, when dry, air fills the interstices and now the situation is significantly different (N_1 = 1.00; N_2 = 1.3-1.5). Even at perpendicular incidence,[7] approximately 4% of the total light is reflected back to the observer. The net result is that most of the illumination falling on the pileus is diffusely reflected back to the observer before preferential spectral absorption from the more deeply buried pigmented hyphae can occur.

This unusual optical property has not been observed in any other Hygrocybe. The observed greenish-yellow colors appear to be associated with those areas of the pileus which were occluded by other pileii or debris.

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[7] In the special case of perpendicular incidence, the reflection, R, from each surface, is given by: $R = \frac{(N_2 - N_1)^2}{(N_2 + N_1)^2}$ where N_1 = index of refr. of first med. N_2 = index of refr. of 2nd med.
As the angle of incidence deviates more and more from 90°, the reflection increases. A complete treatment of this optical aspect is given by Wood (1934, p. 406-412).

APPENDIX D

CLASSIFICATION OF HYGROPHORUS ACCORDING TO HESLER AND SMITH (1963)

Genus *Hygrophorus*
- subgenus *Pseudohygrophorus*
- subgenus *Hygrophorus*
  - section *Hygroaster*
  - section *Amylohygrocybe*
  - section *Hygrotrama*
  - section *Hygrophorus*
    - subsection *Hygrophorus*
      - *H. speciosus* var. *speciosus*
    - subsection *Camarophylli*
  - section *Camarophyllopsis*
    - subsection *Camarophyllopsis*
      - *H. pratensis* var. *pratensis*
    - subsection *Microspori*
  - section *Hygrocybe*
    - subsection *Psitticini*
      - series *Puri*
      - series *Inolentes*
      - series *Psitticini*
        - *H. psitticinus* var. *psitticinus*
        - *H. laetus*
        - *H. nitidus*
        - *H. subminitulus*
    - subsection *Punicei*
      - series *Punicei*
        - *H. puniceus*
        - *H. flavescens*
    - subsection *Hygrocybe*
      - series *Conici*
        - *H. marginatus* var. *concolor*
        - *H. marginatus* var. *marginatus*
        - *H. acutoconicus* var. *acutoconicus*
        - *H. cuspidatus*
        - *H. conicus*
      - series *Hygrocybe*
        - *H. purpureofolius*
        - *H. turundus* var. *sphagnophilus*
        - *H. cantharellus*
        - *H. miniatus* var. *miniatus*
        - *H. strangulatus*
      - series *Coccinei*
        - *H. coccineus*
        - *H. parvulus*