Comparative Color Reaction of *Amanita* Spores with Lugol's and Melzer's Iodine

Patrick Verdier

Pvrfd1@yahoo.com

ABSTRACT

Representative amyloid *Amanita* spores in sections Lepidella, Amidella, Phalloideae and Validae and inamyloid spores in sections Amanita and Vaginatae were examined. Spores developed similar color reactions with both Lugol's and Melzer's iodine in separate cellular compartments: A blue color reaction in the spore wall of amyloid species, and a generally yellow color reaction in the spore cytoplasm (sporoplasm) of all species. The relative contributions from each compartment determined the overall observed color, which varied according to iodine concentration, the presence of chloral hydrate, and time. Chloral hydrate inhibited cytoplasmic staining in both amyloid and inamyloid spores With Lugol's iodine, amyloid spores developed and retained a distinctly green color reaction. With chloral hydrate, amyloid spores developed variable color profiles ranging from green to gray and eventually to blue. Langeron's Melzer reagent developed a blue color more readily than the original 1924 formula due to the reduced iodine concentration in the modified formula. Amyloid spore wall fragments and sporoplasm free spore ghosts developed a blue only color profile with all iodine solutions. Melzer's iodine, and by extension chloral hydrate, was not required to observe the expected blue reaction of the spore wall as this reaction can be generated with aqueous iodine solutions by various simple means.

Keywords: Amanita, spore, Iodine, amyloid, color reaction, Melzer's, Lugol's, spore wall, sporoplasm, Langeron

INTRODUCTION

The purpose of this paper is to:
A. Examine the iodine color reactions of *Amanita* spores in both amyloid and inamyloid species.
B. Provide a method whereby these color reactions can be described and quantified in a useful and repeatable manner.
C. Describe quantitatively the differences between the Melzer's and Lugol's color reactions.
D. Examine the role of chloral hydrate in the color forming process.
There is a continued debate as to which iodine solution is best when evaluating the color reaction of Basidiomycete spores, generally, but not exclusively in relation to the white-spored Agaricales (25,40). These reactions are referred to as either amyloid (a blue reaction) or dextrinoid (a red reaction). The terminology and descriptions have evolved over time (blue coloration, amyloid like substance, pseudo-amyloid, etc.) based on the contemporaneous understanding of these reactions (3,6,10,13,20,22,31,37,38). In ascomycetes, the situation is more complex with additional terms (hemiamyloid, euamylod) based on differential color reactions (blue, red or mixtures of both) observed in Lugol's iodine, and a masking effect of the red reaction observed in Melzer's iodine, either by pretreatment (generally potassium hydroxide, KOH) or the use of different concentrations of iodine (3,20,37). The reagents used in evaluating these color reactions are one of various formulations of Lugol’s iodine or Melzer’s solutions (3,4,12,13,20,21,22,24,32,37,40).

It was almost exactly100 years ago that Melzer published his now famous iodine formulation (32). The original intent was to provide an improved method to clarify the fine structure of surface ornamentations in spores of *Russula* species based on previous work by Maire in 1910 (28). Maire did not use iodine, but was aware of its effects and prophetically wondered if the iodine reaction could be useful in systematics.

Gilbert and Kühner in 1928 (13) pioneered the widespread use of iodine solutions for study of Basidiomycete spores as a taxonomic tool in their studies of *Amanita*. The key to Melzer’s formula was the addition of large amounts of chloral hydrate, which “clears” the spores and increases depth of field. This is due to the high refractive index of chloral hydrate, which,

approximates more closely the refractive index of the spore content. When refractive indices are similar, objects appear more or less transparent (often described as homogenized). These optical effects were well known before this time and quite familiar to Langeron who published the first edition of his “Précis de microscopie” in 1913 (23). This was a foundational and highly detailed manual of microscopy instrumentation, optics, illumination sources, sample preparation, histological techniques, and histochemical stains. The final (7th) edition of this work was published posthumously in 1949.

The first 1913 edition lists multiple examples of stains containing chloral hydrate and iodine, but never together in the same formula. In 1945, Langeron published an altered formula for Melzer's iodine in his “Précis de mycologie” (24). In his revised formula he decreased the iodine concentration and increased the potassium iodide to iodine ratio. Langeron had extensive experience with iodine solutions listing numerous formulations among them “Lugol faible” (weak), “Lugol forte” (strong), and “Lugol ordinaire”. He no doubt reduced the iodine concentration because he considered the original formula to be unnecessarily strong, and increased the iodide ratio to get (just over) a 2 to 1 stoichiometric ratio of iodide to iodine. There have been various Melzer’s formulations used over the years. Baral (3) lists the effects of multiple formulations containing various proportions of iodine, potassium iodide, chloral hydrate, and various other ingredients.

A review of Langeron's 1945 edition in the “Société Mycologique de France” states that Langeron was never a great proponent of the study of higher fungi. In France, mushrooms were (and still are) considered important mostly in relation to their edibility (or toxicity). The term “champignon” was and is still used indiscriminately to describe all fungi. Langeron, a medical mycologist, grouped the study of “champignon” with the study of bacteria in terms of sample preparation and histological technique since he was mainly interested in their relationship to human disease. He eventually addressed the higher fungi in more detail in the 1945 “Précis de Mycologie”. As an interesting side note, the Société Mycologique also noted that the 1945 "Précis de Mycologie" was truncated due to paper shortages and that all of Melzer’s original works were regrettably destroyed by the Germans during the war.

As previously stated, it was Gilbert and Kühner who pioneered the widespread use of Melzer's iodine (using the original formula) for their studies of spores of *Amanita* species. The original formula, or Langeron's modification, have been used virtually exclusively ever since for studies of the Agaricales. Mycologists studying ascomycetes (including lichens) have been more flexible in terms of iodine solutions (having used them for many years) but for the white-spored Agaricales, Melzer's is considered essential to this day in order to develop the expected "blue" or "red" reaction in fungal spores. Certain treated spores are indeed unequivocally blue (*Russula, Lactarius* etc.), but others (*Amanita* species) are often described as some hue of gray, with perhaps subtle bluish tints. Gilbert (12) described the spores as "colorless" to gray. Kühner (22) recommended daylight illumination in order to observe the effect. This is no surprise since daylight contains far more blue light than the "yellow" artificial illumination of the time. Aqueous iodine does not seem to impart a blue coloration even in *Russula* spores where the reaction is often described as "black."

Regarding the chemical constituents responsible for these reactions of Basidiomycete spores, research indicates that a short chain amylose (30 to 50 subunits) is responsible for the amyloid (blue) color reaction. The red (dextrinoid) reaction has been attributed to iodine binding to non-carbohydrate glycine betaine compounds (6,10,30).

These color reactions closely resemble the iodine color reactions of the two main components of starch: amylose which stains blue, and amylopectin which stains variously red, red-brown, or red purple, green etc. (1,2,18,35,36,41,42). Dextrin, a breakdown product of starch and glycogen also stains red (or red brown) with iodine, thus the derived term dextrinoid (3). The color reaction of starch with iodine was discovered in 1815 by Colin and Gauthier (38) and has been extensively studied ever since to characterize the carbohydrate contents of starchy foods as well as the physical chemical nature of starch itself (1,2,8,18,35,36,41,42).

Chemically, amylose consists of long chain polymers of glucose molecules, whereas glycogen and amylopectin consist of shorter highly branched chains forming overall tree like structures. (2,8,41). It is generally understood that both forms can produce secondary helical structures and that iodine intercalates within the helix (8,18,35,36,41,42). Spectroscopic studies (1,2) show that

very short chains (0-7 monomers) produce no color reaction, long chains (35+) produce the characteristic blue color, and intermediate chains (7-35 monomers) produce red, brown, and other intermediate colors. Recent physical chemistry studies implicate several iodine/iodide anion combinations as responsible (14,32 38). The structure of helical starch is such that 6 monomers create one revolution with each revolution a potential placeholder for 1 iodine atom, subject to a minimum chain length. Triiodide (and other polyiodides) are frequently invoked as generating these colors (1,8,18,41,42); however recent physical chemistry studies suggest that the blue reaction is the result of specific iodine triiodide complexes held in place and stabilized within the carbohydrate helix (42). The blue reaction is not restricted to starch, but can be generated by other helical carbohydrate compounds in ascomycetes (Ascal walls, Ascal plugs, subhymenial gels), as well as other non-carbohydrate helical molecules (2,42).

These colors are due to the specific light absorbing properties of charge transfer (CT) complexes (16,42). These complexes are the result of weak, generally reversible electrostatic interaction between atoms and molecules that involve sharing of electrons between atomic orbitals. Photons within a particular energy range can be absorbed if their energy lies within the energy range difference of the shared orbitals. The final observed color with standard brightfield microscopic illumination is the color of the reference illumination (generally “white”) minus the sum of all absorbed colors, from all sources. Carotenoids are well studied examples of CT complexes. The caroteine molecules fold and form specific intramolecular charge transfer complexes that absorb blue light and some green light, resulting in a “leftover” orange color. When carotenoids interact with triiodide, a new, highly specific intermolecular charge transfer complex is formed that absorbs blue and red light resulting in a green color (19). Light absorption of CT complexes is not limited to visible wavelengths but can extend into the deep UV and IR as well. Amyloid (blue) reactions absorb red and green light, and dextrinoid (red) reactions absorb blue and green light, both in various proportions. The remaining, non-absorbed light determines the final color, described subjectively as either “blue,” “red,” or some variation of these colors.

Currently, amyloid/dextrinoid reactions in the Agaricales are visualized using one of several methods, including mixing the spores directly with various iodine solutions on glass slides and observing the color reaction macroscopically, putting drops of reagent directly on spore prints, or

observing the spores or gill sections under the microscope in small amounts of reagent. Results are reported subjectively as positive (J+) (from the German word for iodine-Jod), negative (J-), weakly positive, positive after a certain period of time (or after a particular treatment), dextrinoid (red), weakly dextrinoid, etc. In addition, the specific colors are variously described as blue, brown, red, black, gray, blue gray, and other color combinations (12,13,14, 20, 22, 32,43).

These descriptions are invariably subjective and therefore the purpose of this paper, as previously stated, is to provide a method whereby these color reactions can be described and quantified in a repeatable and useful manner, to describe the differences between the Melzer's and Lugol's formulations, and to examine the role of chloral hydrate in the color forming process.

MATERIALS AND METHODS

Over the summers of 2022 and 2023, spore prints were acquired from a variety of *Amanita* species collected in Rhode Island, Pennsylvania, Connecticut and Florida. The species studied included *A. amerirubescens, A. brunescens, A. flavoconia, A. lavendula, A. volvata, A. fulva, A. bisporigera, A. frostiana. A. onusta, A. cinereoconia, A. whetstoneae A. albocreata and A. muscaria* among others. *Russula* spores of various species were also examined for comparative purposes. Spore prints were collected on aluminum foil, individually labeled and stored in zip lock bags for future evaluation. In some cases, spore prints were examined immediately after collection to determine the effect, if any, on time as a factor in the observed color profiles.

Spores were carefully collected from the prints with a flattened toothpick and mixed with a 10 µL solution of either Lugol’s solution at various iodine concentrations or one of several formulations of Melzer's reagent (Table1). In certain cases, spores were mixed with 10 µL drops of water on a slide, or a coverslip and allowed to dry before staining. In another set of experiments, spores were mixed with 10 µL of dilute Lugol’s solution (0.625%-1% final iodine concentration), allowed to dry, and subsequently rehydrated with water, chloral hydrate, or immersion oil prior to observation. Spores previously stained with iodine solutions were observed after “washing out” by infiltrating water or chloral hydrate from the edge of the slide

while wicking the solution from the other side of the coverslip. A limited number of experiments were performed with spores treated with iodine in a 50% glycerol/water solution. Various spores were used for colorimetric measurements, and in certain cases intermixed with inamyloid spores to serve as internal negative control. *Russula* spores were also examined for comparative purposed. *Amanita amerirubescens* spores were used for the detailed quantitative colorimetric analysis.

Table 1. Formulations of iodine solutions. Percent iodine is listed with two values to reflect nominal and estimated actual concentration. The estimated concentration is due to the fact that 100 g of chloral hydrate and 100 ml water yield less than 200 ml of final volume. KI to I ratio is listed by weight and by molar ratios. Aqueous iodine solutions were prepared by diluting the Lugol's stock solution to appropriate final concentrations (generally 0.6 percent)

iodine solution	I	KI	Chl.hyd.	Water	% iodine	KI/I ratio
Lugol's stock	5g	10g	0	100	5	2 (1.3)
1924 Melzers	0.5g	1.5g	20g	20ml	1.25-1.5	3 (1.97)
Langeron Melzer's	1.5g	5g	100g	100ml	0.75-0.9	3.33 (2.2)

All spores were visualized either immediately, after an interval of a half hour, or after an interval of 24 hours, and in some cases after an interval of several days. Spores mounted in aqueous Lugol's solution were visualized over a shorter period of time due to the more or less rapid evaporation of this entirely water-based preparations, and in some instances, spores treated in Lugol's were kept overnight in a humidity chamber prior to observation or sealed with nail polish for longer term observation. In certain cases, cross sections of the gills were also examined in order to observe the overall staining pattern of the hymenial layer.

All microscopic visualization was performed with a Nikon Optiphot microscope (Nikon Instruments) equipped with a 1.4 NA condenser (effective NA 0.95 in air) and a Plan Apochromat 40X air (NA 0.95) or a Plan Apochromat 10X (NA 0.45). In all cases, the field stop was focused and centered at 50% of the field of view and the aperture stop was set to just inside the back aperture (as seen through the microscope tube with the eyepiece removed), consistent with standard Köhler illumination practice. In addition, the correction collar on the 40X

objective was set individually for each specimen examined, and No. 1.5 coverslips were used throughout as specified by the manufacturer.

Images were acquired with a Diagnostic Instruments Spot Insight 4K monochrome camera (model 14.5 w/o IR filter), and IPLab (V3.2) Macintosh software (BD Biosciences). In all cases, images were acquired in 12 bit mode (4096 gray levels) with no offset. The illumination source was a Lumencor Lida 3 color LED light source (Figure 1) with peak LED wavelengths of 630 nm (red), 532 nm (green), and 435 nm (blue) with ground glass diffusers provided by the manufacturer. Color images were acquired sequentially with red, green and blue illumination and merged to form 12 bit color composites. All elements of the optical path were free from of any filters, polarizers, phase rings, Wollaston prisms etc. In certain cases, spore preparations were imaged at low magnification using a flatbed scanner.

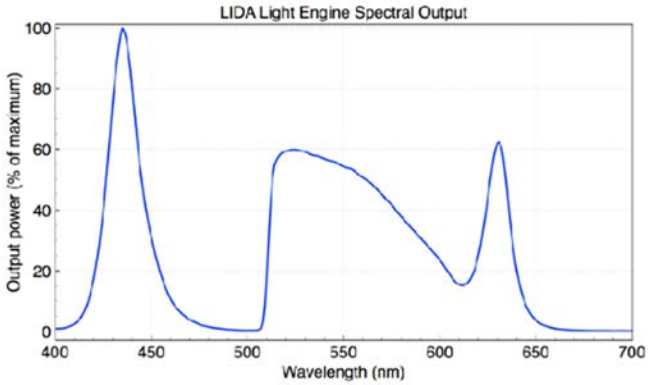

Figure 1. Spectral output of Lumencor Lida with peaks at 630 (red), 532 (green) and 435 nm. (blue).

For qualitative images, field of view was restricted to an area of circa 80 X 80 microns for the 40X objective. For quantitative images, field of view was restricted to a small region (22 X 22 microns) about the center of the optical axis in order to avoid any unevenness in the field of view. Ideally, focus was set at the center of the spore with the hilar appendix in the plane of focus. White balance was set by adjusting the exposure time for each channel such that the final background (white) values were equal, at approximately 85% of the full range of the camera (12 bit gray value 3500) in order to avoid any saturation.

Selective colorimetric analysis was performed on the iodine solutions as well, either by mounting 10 µL solutions on a coverslip or creating an approximately 170 micron chamber using 2 coverslips as spacers with a third coverslip on top. To ensure consistent measurements, the initial RGB exposures were first set with a10 µL water mount, and consistent exposure times were used subsequently.

All image processing and analysis was done using ImageJ (Fiji version https://www.nature.com/articles/nmeth.2019). As a first step, the white balance for each image was refined and set to a standard value of 3500 by multiplying each channel by the proportional deviation from that value within a background region. Raw data RGB plot profiles were generated by drawing a line across one or several spores, and running a color plot profile macro. RGB, values were measured from multiple spores in several replicates for each treatment and subsequently averaged. All measurements were performed by drawing a small region in the central area of the spore, carefully avoiding refractive edges. From the averaged data, percent transmission value (V) per RGB channel was calculated by V/3500*100. In order to graphically represent the observed colors, Image thumbnails were generated by creating 12 bit RGB images with the values from the averaged data, and linearly converting to 8 bit RGB.

RESULTS

As shown in Figure 2, all solutions were nearly, but not entirely, transparent in the red channel, moderately absorptive in the green channel and heavily absorptive in the blue channel in proportion to the iodine concentration and the length of the light path. The subjective visual color ranged from yellow to orange to deep orange brown, and eventually opaque (no appreciable light transmission by eye). Solutions with similar iodine concentrations (B and C, See Table 1) showed similar color profiles independent of potassium iodide concentrations or the presence of chloral hydrate. The water solution is scaled at 85% of full range, as are all subsequent images. The slightly irregular line profiles reflect pixel to pixel variation (shot noise) present in all images.

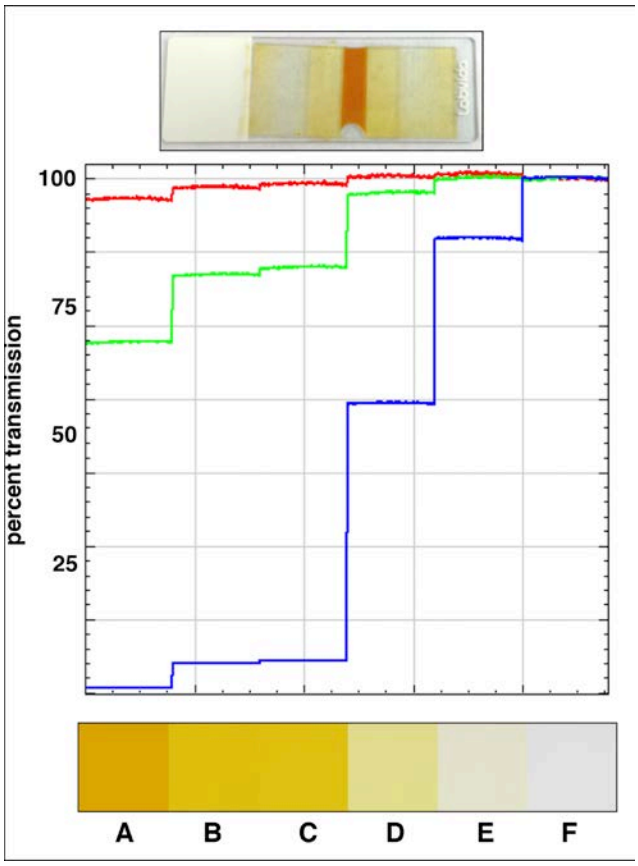

Figure 2. Optical transmission properties of iodine solutions.: X axis: original 200 X 200 pixel images of iodine solutions from the center of the microscope's optical axis. Y axis: Percent transmission of incident RGB light. Top: Image of slide with 170 micron chamber (approximate measurement based on coverslip thickness). Bottom: A. 1924 Melzer in 170 micron light chamber, B. Lugol (1% iodine) in 170 micron chamber, C. Langeron Melzer in 170 micron chamber, D.1924 Melzer, 10 µL with coverslip, E. Langeron Melzer, 10 µL with coverslip, F. Water.

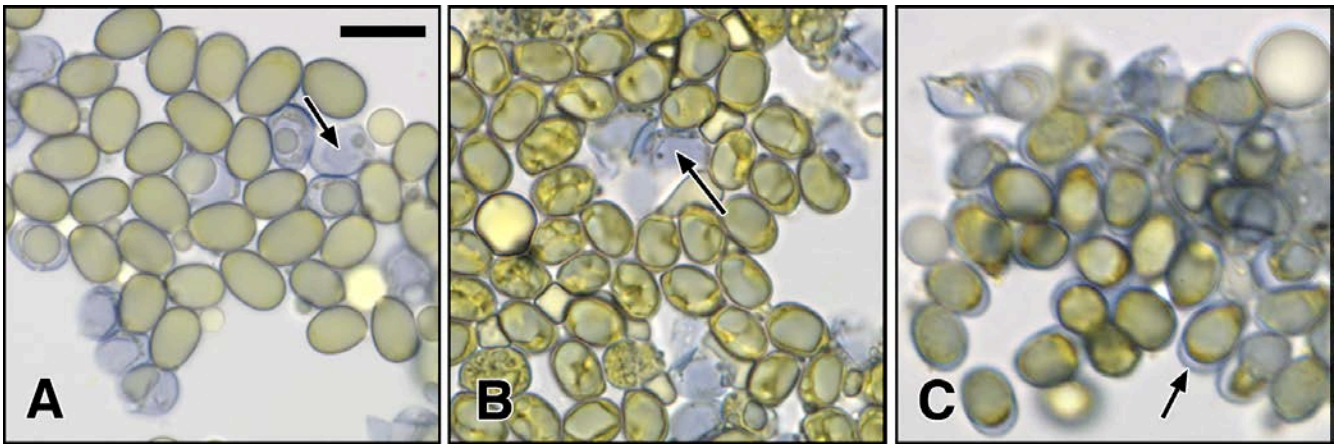

Figure 3. A. Typical color reaction of *A. amerirubescens* immediately upon staining with Melzer's original 1924 formula. B. Typical Lugol's (0.625% iodine) reaction immediately upon staining. C. Lugol's (0.625% iodine) reaction after several days. Scale = 10 microns.

In order to prevent evaporation, cover glasses were sealed with nail polish (Figure 3). The *A. amerirubescens* spores developed a distinct olive-green coloration as an immediate reaction upon staining with Melzer's original 1924 formula (Fig. 3A) while the spore wall fragments (Fig. 3A, arrow) were distinctly blue. For spores treated with Lugol's solution (Fig 3B), the color profiles of the spores were similar to The Melzer's treated spores but subjectively somewhat "dirtier". Spore wall fragments (Fig. 3B, arrow) remained distinctly blue. After several days in Lugol's solution (Fig. 3C), the sporoplasm eventually shrank away from the spore wall (Fig. 3C, arrow), and both color reactions (blue and olive green) could be seen simultaneously in individual spores. The blue color (red absorption) was clearly present over the entire spore wall, but only apparent where the cytoplasm shrank away from the spore wall.

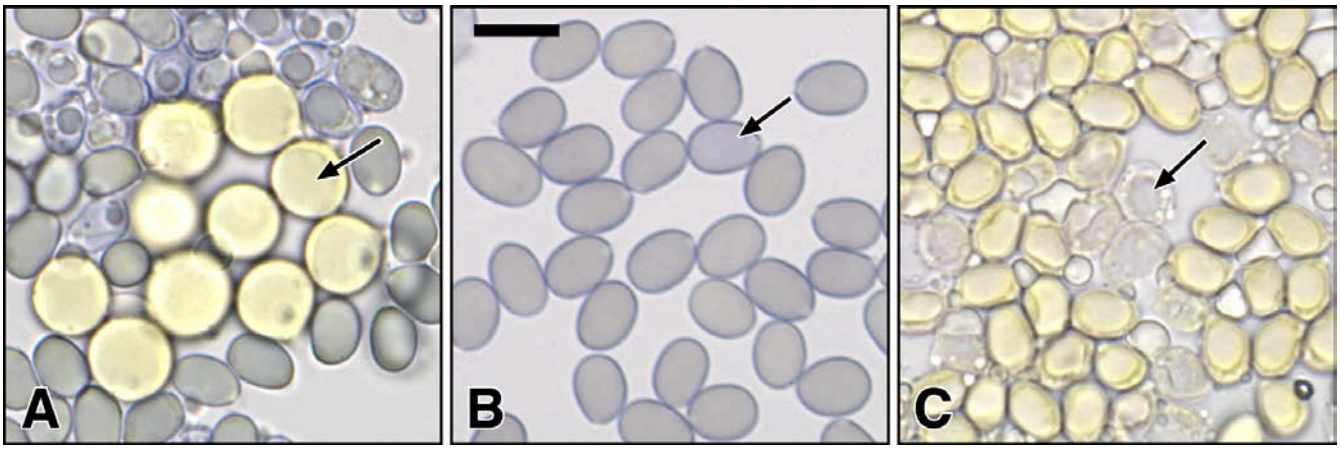

Figure 4. Staining of *A. amerirubescens* spores with *A. fulva* spores (arrow) as negative control. A. Typical Langeron's Melzer reaction immediately upon staining. B. Langeron's Melzer after 24 hours. C. Spores treated with 0.5% iodine in 50% glycerol. Scale = 10 microns.

In Figure 4A, the *A. fulva* spores bound a certain quantity of iodine and were yellow in color. The *A. amerirubescens* spores developed a color similar to the 1924 formula (Fig. 2A), but subjectively with diminished intensity. Blue spore walls are evident in the many collapsed or otherwise damaged spores at the top of the image. In Fig. 4B, the spore content became homogenized to a neutral gray appearance, and distinctly blue (arrow) in certain cases, more readily apparent by eye. The blue color became more evident over time, and more or less rapidly with increased ambient temperature and local spore concentration. In Fig. 4C, all color reaction was completely suppressed in the spore wall fragments (arrow), while the color profile of the intact spores approximated that of amyloid negative spores.

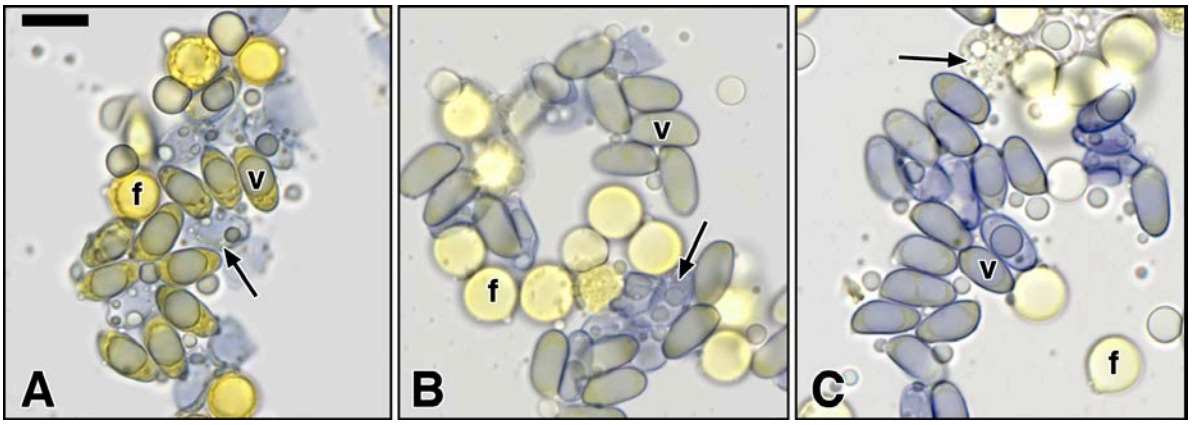

Figure 5. Mixed spore preparation, of *Amanita frostiana* (f) and *Amanita volvata* (v): A. Lugol's 0.625% iodine B. Melzer's 1924 formula C. Langeron Melzer's. Arrows in A and B indicate blue staining of spore ghost void of sporoplasm. Arrow in C indicates unstained inamyloid spore ghost. The relative staining of the inamyloid *Amanita frostiana* spores decreases from A to C while the relative staining of the amyloid *Amanita volvata* spores transitions from olive green to distinctly blue. Scale = 10 microns.

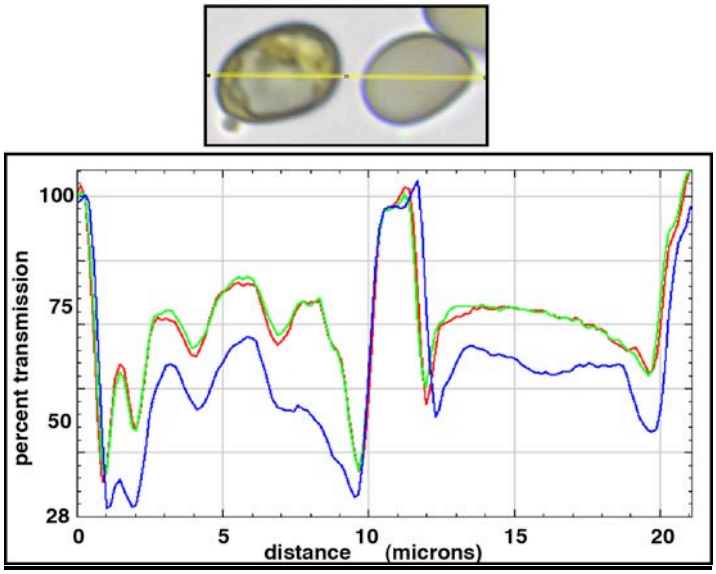

Figure 6. RGB line profiles through a combined image of individual *A. amerirubescens* spores, with 0.65% Lugol's iodine (left spore) and Langeron's Melzer (right spore): X axis: distance in microns. Y axis: percent transmission of incident light. Red, green and blue transmissions are indicated by the red, green and blue line profiles, respectively.

Peak-to-peak variability of the Lugol treated *A. amerirubescens* spores is far greater than the Langeron treated *A. amerirubescens* spores but the overall average levels are similar, as shown in Figure 6. In relation to the iodine solutions themselves (Figure 2), the red transmission is substantially reduced while the blue transmission is substantially increased. Red and green transmission are almost equivalent, with green exceeding red in certain portions of the profile, leading to an overall observed "dirty" green appearance. The decreased peak-to-peak variability in the Melzer's iodine profile is a 2-dimensional representation of the 3-dimensional smoothing (homogenization) effect as seen by eye.

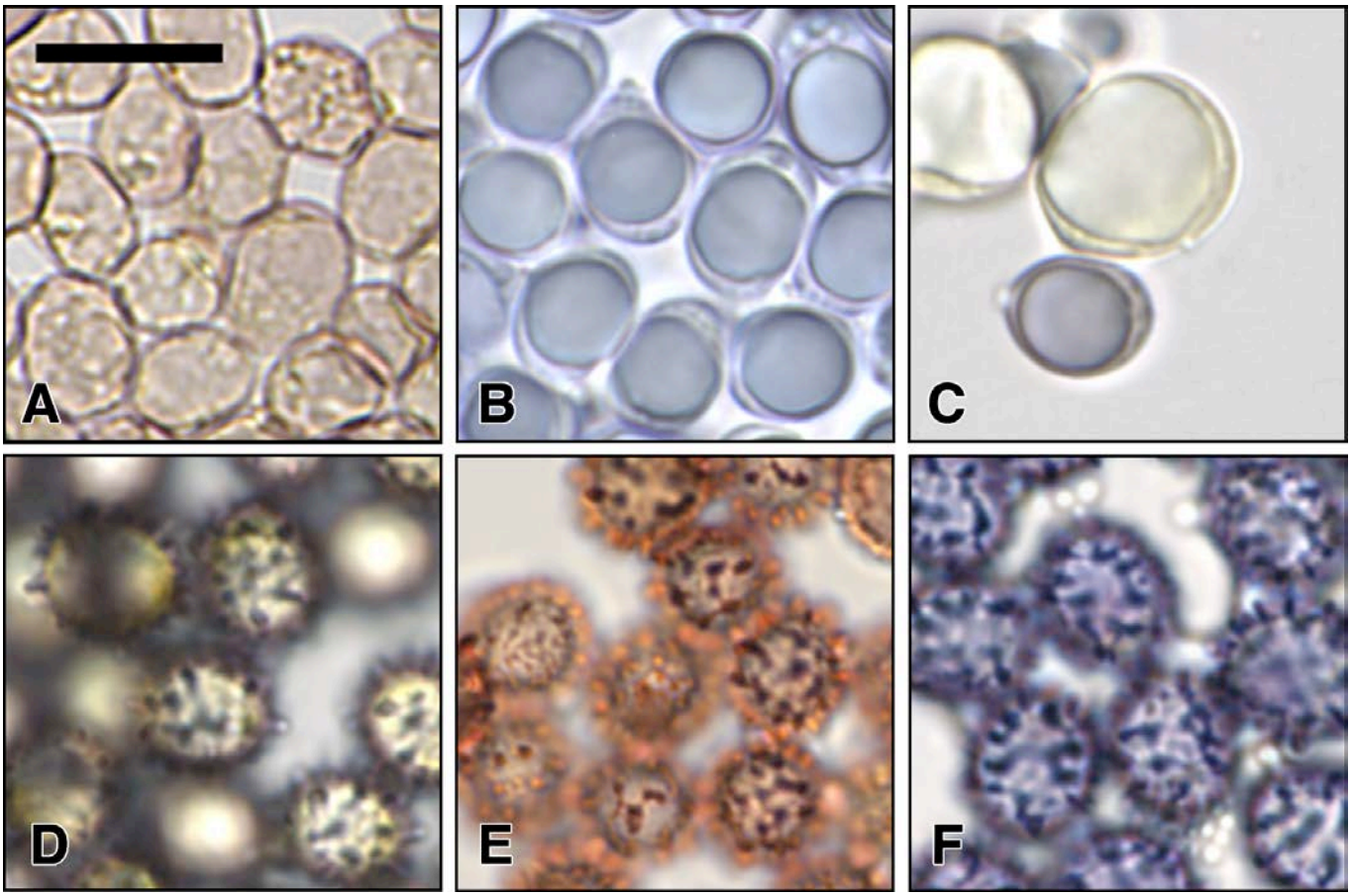

Figure 7. Spores of *Amanita amerirubescens* and *Russula* species treated with Lugol's iodine (0.65%), dried and subsequently mounted in either immersion oil, or rehydrated and mounted in water: A. Treated *Amanita* spores dried, and mounted in immersion oil B. Treated *Amanita* spores dried, and mounted in water C. Treated *Amanita* spores with internal *Amanita fulva* control dried, and mounted in water D. *Russula* spores in Lugol's solution E. *Russula* spores

treated with Lugol's iodine, dried, and mounted in immersion oil F. *Russula* spores treated with Lugol's iodine, dried, and mounted in water. Scale = 10 microns.

Upon drying after Lugol's treatment (Figure 7), *Amanita* spores developed a distinct red color, which persisted when mounted in a non-polar solvent, in this case, immersion oil (Fig. 7A). When rehydrated with water, an immediate blue color developed (Fig. 7B), with only a slight residual yellow color left in the *Amanita fulva* inamyloid spores (Fig. 7C). With Lugol's iodine, the *Russula* spore ornamentations appeared dark blue trending toward a more neutral dark gray (Fig. 7D). When dried and mounted in immersion oil (Fig. 7E), the ornamentations developed a reddish color similar to that of the *Amanita* spores but with far greater intensity. When rehydrated with water (Fig. 7F), the sporoplasm staining disappeared, and the ornamentations developed an intensely blue color.

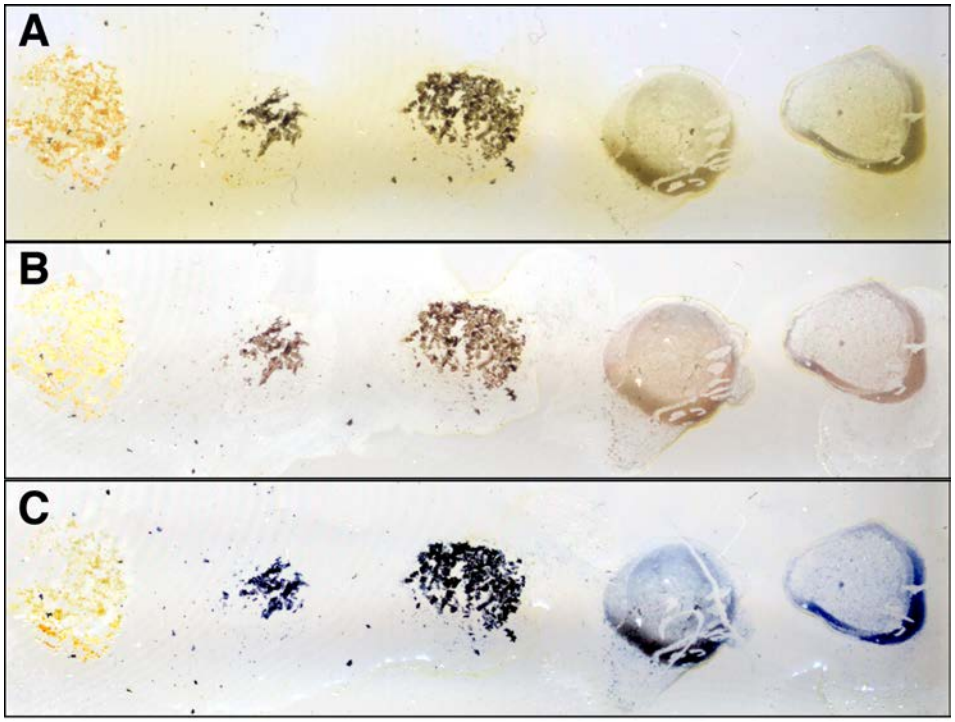

Figure 8. Spores treated with A. aqueous iodine, B. dried and C. rehydrated as imaged on a flatbed scanner: From left to right: *Amanita fulva, Amanita lavendula, Amanita brunescens, Amanita flavoconia,* and *Amanita amerirubescens*.

In Figure 8, all four examples of amyloid spores show identical, or very similar color profiles; namely dirty “olive green” 10 minutes or so after staining, distinctly reddish when dried, and distinctly blue when rehydrated. *Amanita fulva* develops a yellow orange stain subjectively similar to the iodine solutions themselves. All species tested in the four amyloid positive sections of *Amanita* develop similar color profiles.

Table 2. Summary data of mean raw data and percent RGB transmission of *Amanita amerirubescens* spores with various treatments. Data and corresponding RGB thumbnails are arranged in order of increased blue transmission.

	1	2	3	4	5
	TREAT.	TN	RED	GREEN	BLUE
1	LUGOL 2.5%		2392 -68%	1824 -52%	334 -9%
2	LUGOL 0.625%		2502 -71%	2587 -74%	2154 -61%
3	1924 10 MIN		2653 -76%	2706 -77%	2154 -61%
4	LANG 10 MIN		2747 -78%	2769 -79%	2489 -71%
5	1924 24 HR		2734 -78%	2742 -78%	2700 -77%
6	LANG 24 HR		2761 -76%	2820 -80%	2795 -80%
7	REHYD WATER		2664 -76%	2742 -78%	3037 -87%
8	REHYD CHL.HYD		2996 -86%	3041 -87%	3326 -95%

The staining results for *Amanita amerirubescens* spores undergoing various treatments is given in Table 2. At high iodine concentrations (Table 2, row 1), blue transmission was very low, but increased rapidly (Table 2, row 2) from 9% to 61% in aqueous formulations with lower iodine concentrations. In solutions with chloral hydrate, relative blue transmission increased as a function of time, and preferentially with Langeron's formulation (Table 2, rows 4 and 6). Spores appeared green where green trended higher than red (Table 2, rows 2, 3, and 4), and neutral gray when all colors approached parity (Table 2, rows 5 and 6). A distinct blue color became apparent when blue transmission exceeded that of red and green (Table 2, rows 7 and 8). Increased ambient temperature appeared to increase the rate of transition from green to blue in the solutions containing chloral hydrate.

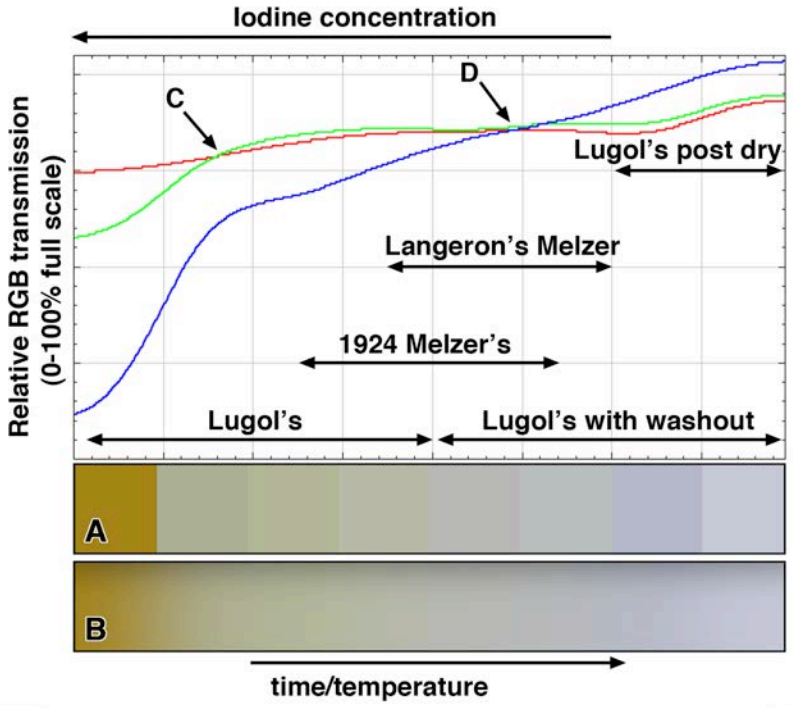

Figure 9. Synopsis of all observed color reactions with Melzer's and Lugol's iodine (relative iodine concentration decreases from left to right) A. Original thumbnails from Table 2 arranged in order of increased blue transmission B. Gaussian blur of thumbnail images used to generate intermediate colors. C and D are color profile inflection points.

The color profile in the Figure 9 plot shows two inflection points: One at Fig. 9C where the observed color became distinctly green, and one at Fig. 9D where the observed color became

distinctly blue. Inflection point Fig. 9D occurs where red, green and blue are equivalent, and corresponds to the observed "gray" color as frequently described in the literature. Langeron's Melzer's developed a blue color more readily than the 1924 Melzer's although both formulations eventually did so. Treatment with Lugol's iodine at high iodine concentrations (left of Fig. 9C) show rapid increase of blue absorption similar to that of native iodine solutions.

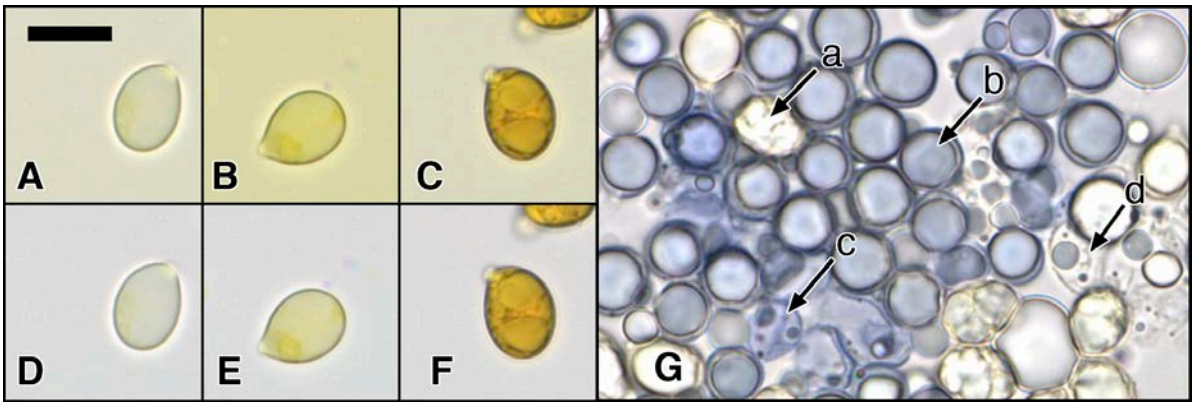

Figure 10. A through F: *Amanita muscaria* spores with and without internal background correction: A. In Langeron's Melzer's solution, white balanced with water. B. In the original Melzer's solution, white balanced with water. C. In Lugol's iodine (0.625%), white balanced with water. D. In Langeron's Melzer's solution, white balanced with local background. E. In the original Melzer's solution, white balanced with local background. F. In Lugol's iodine (0.625%) , white balanced with local background. G. *A. muscaria* and *A. brunecens* spores treated with Lugol's iodine (0.625%) and subsequently washed out with water: a. Intact *A. muscaria spore*, b. intact *A. brunecens spore,* c. *A. brunescens* spore ghost, d. *A. muscaria* spore ghost Scale = 10 microns.

Langeron's Melzer (Figure 10A) and Lugol's iodine (Fig. 10C) solutions showed nearly identical background values while the original 1924 Melzer's solution (Fig. 10B) was distinctly more intense. The color intensity of the spores themselves was least intensely stained in Langeron's Melzer (Fig. 10D). In all cases, spores accumulated iodine stain well in excess to that of the native iodine solutions. The inamyloid *A. muscaria* spores (Fig. 10Ga) showed a highly reduced level of iodine staining and the refractive contents generated intensity levels above that of the incident illumination. Amyloid spore (Fig. 10Gb) and amyloid spore ghosts (Fig. 10Gc) retained a blue only color reaction while inamyloid spore ghosts (Fig. 10d) were nearly transparent.

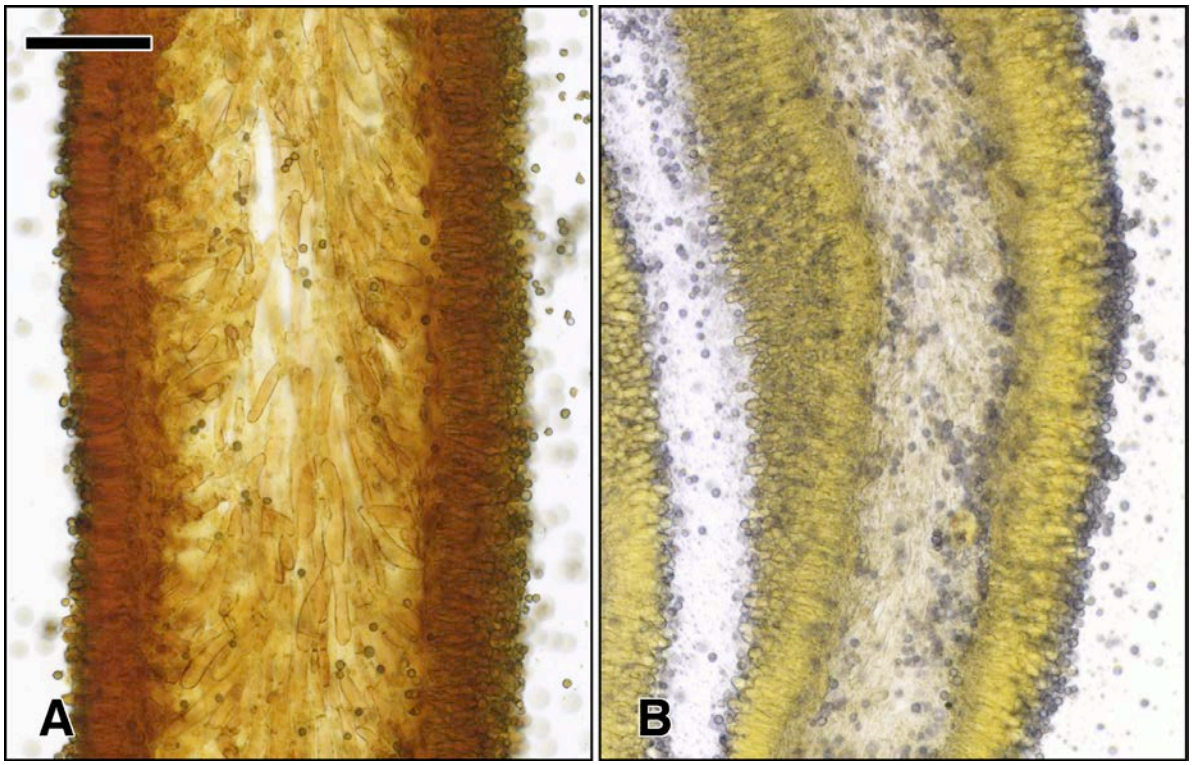

Figure 11. Longitudinal gill sections of *Amanita lavendula* A. Gill section treated with 0.5% Lugol's iodine demonstrating a dextrinoid type reaction in the hymenial layer. B. Gill section treated with Melzer's iodine showing a blue gray reaction in the spores, and no reaction in the hymenial layer. Scale =50 microns

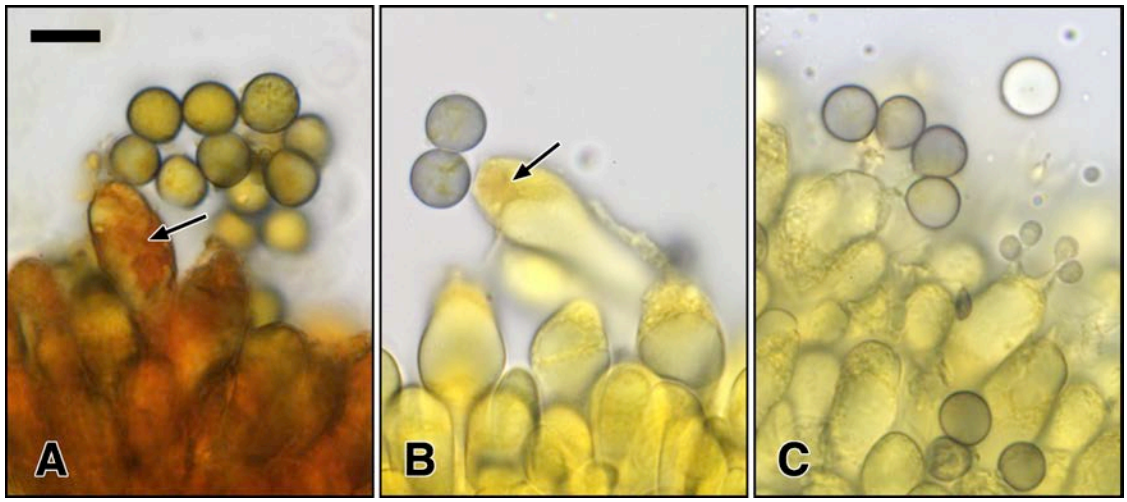

Figure 12. Basidia and spores of *Amanita lavendula* A. Basidia and spores treated with 0.5% Lugol's iodine. Arrow indicates a dextrinoid type reaction within a basidium B. Basidia and spores treated with the original 1924 Melzer's formulation. Arrow indicates a weak dextrinoid

type reaction within a basidium. C. Basidia and spores treated with Langeron's Melzer's showing no dextrinoid reaction. Scale = 10 microns.

DISCUSSION

The amyloid reaction in the white-spored Agaricales is defined as “blue”, but as noted in the introduction, this color is not always unequivocally observed. The initial color of the spores of all amyloid positive *Amanita* spores examined here was, with certain exceptions, distinctly green. This color can be easily observed without a microscope by mixing the spores in small volumes (10 μL) of Lugol’s solution at appropriate concentrations and allowing the mixture to stand still for 10 minutes or so. The solution will eventually clear, presumably from a combination of iodine incorporation and sublimation of excess iodine. The color of the spore mass then becomes unequivocally green or “dirty green” as seen by eye (Fig. 8) as well as under the microscope. Microscopic observations and colorimetric analysis confirm this color both with Lugol’s and Melzer’s formulations. As shown in Figs. 3, 4, and 5, the staining intensity is greater with the original 1924 Melzer formulation as opposed to the Langeron modification.

As noted by all investigators, and demonstrated from the above results, the blue portion of the color reaction in amyloid *Amanita* spores is restricted to the spore wall. This reaction is seen in both Melzer’s and Lugol’s formulations in the spore wall and spore wall fragments (Figs. 3, 4 and 5). In this regard it is useful to make a spore preparation directly on the coverslip. Upon drying, a portion of the intact spores, immature spores and spore wall fragments adhere to the coverslip and do not drift as when seen in the microscope. There is invariably a population of “ghost spores” (spore wall only) that stain obviously blue with or without chloral hydrate. The blue reaction may seem subjectively weak, but is in fact quite strong, considering the nanometer thickness of the spore wall as compared to the micron thickness of the spore itself. The content of the spore itself generates a separate color reaction, and it is the combination of these two reactions that generates the final observed color. As summarized in Table 2 and Fig. 9, color profiles change with iodine concentration and time (and, from personal observations, also with temperature as well as relative spore density), but the initial color profile, immediately upon staining is consistently some shade of green, with or without chloral hydrate. Despite the green

color, the spore wall stains “blue” in all cases (Fig. 3) whether or not this color is actually observed directly. In sealed preparations with Lugol’s iodine, the cytoplasm gradually separates from the spore wall over time (Fig. 3C) and the blue color of the spore wall and overall green color of the spores can be visualized simultaneously.

Iodine solutions in nonpolar solvents have typical absorption peaks in the green region of the spectrum transmitting mostly red but some blue as well resulting in a purple color. This can readily be observed by dissolving small pieced of iodine in immersion oil. In water the absorption is blue shifted and iodine now has a yellow color but low solubility (16). With the addition of potassium iodide, the solubility greatly increases, because of the formation of triiodide. Triiodide has a strong absorption peak in the near UV which tapers gradually and absorbs additional blue light (16). The result is the characteristic color of iodine as shown in Fig. 2: almost transparent in the red channel, moderately absorptive in the green channel, and highly absorptive in the blue channel. Langeron’s Melzer and 1% Lugol’s iodine have nearly identical absorption characteristics as they are similar in iodine percentage. The nominal percentage of iodine in Langeron’s Melzer is 0.75% but is actually higher as indicated in Table 1 as 100 grams of chloral hydrate and 100 ml of water do not add up to 200 ml of solution, but something considerably less. The KI ratio differences of 2/1 and 3.33/1 appear to make little difference in the transmission properties of the iodine solutions as well as the staining effects, but this may not always be the case at very high or very low KI/I ratios. As indicated in Table 1, the nominal KI/I ratios are different from the molar KI/I ratios, and thus there is a somewhat arbitrary nature to these proportions.

The color of *Amanita fulva* and *Amanita frostiana* negative controls is superficially similar but not necessarily identical to that of a dilute iodine solution. It is clear however, that even the "negative" spores show preferential binding to iodine based on the accumulation of color far greater than that of the background iodine solution (Fig. 10). At high iodine concentrations (Table 2, row 1 and Fig. 9, left side) the reaction is strong enough to emulate a dextrinoid type appearance as described by Leonard (25). In the case of globose inamyloid spores, the true absorption characteristics are difficult to interpret due to a lensing effect which increase the apparent intensity of the spores (particularly in the red channel) above the value of the incident light (Fig. 5C).

Melzer's is said to have an enhancing effect and this may be true in the case of *Russula* and *Macrolepiota* species. However, this is not necessarily an enhancement *per se*, but rather a suppression of subjectively non-specific background staining. This appears to be the case with inamyloid *Amanita* spores (Figs. 5 and 6). Melzer's will completely suppress certain specific staining reactions in ascomycetes (3,4), while enhancing others. As demonstrated here (Figs. 10 and 11) chloral hydrate completely inhibits all dextrinoid type reactions in the hymenial layer of *Amanitas* which can be partially offset by increasing the iodine concentration (by using the more concentrated 1924 Melzers (Fig. 10B). Glycerol also suppresses or enhances staining, sometimes with opposite effects to chloral hydrate (personal observations). Glycerol acts as a plasticizer with amylose and amylopectin by disrupting the native hydrogen bonding and forming new hydrogen bonds with glycerol's three glycerol hydroxyl groups (9). As such, the secondary structure of amylose is altered and can no longer incorporate iodine. As shown in Fig. 4C, glycerol blocks virtually all staining in the *Amanita* spores, and will do so with *Russula* as well to a lesser degree (not shown). This supports evidence for the presence of starch or similar carbohydrate in the spores of species within both genera.

Staining of ascal plugs and ascal walls are not necessarily inhibited by glycerol (personal observations), but may or may not be by chloral hydrate in certain cases as in the hemiamyloid reaction (3,4). This supports the notion that distinct iodine incorporating helical carbohydrates are involved in the various members of the ascomycetes and basidiomycetes, respectively.
A number of alcohols and polyols are know to interact with carbohydrates via hydrogen bonding interactions as well (9), and chloral hydrate, with two hydroxyl groups and very strong hydrophilic properties (allowing very high concentrations in aqueous solutions), may interact in a similar manner. The "enhancing" effects of these hydroxyl groups containing solvents is "enhancing" in that they inhibit certain non-specific reactions, or more importantly, reactions that are not expected or desired by the observer. In this regard, methods are often employed to achieve certain desired reactions such as treatments with strong alkali, heating or even boiling preparations in water or chloral hydrate at unknown but presumably relatively high temperatures (43). The differential reactions after these pre-treatments necessarily reflect some change in native structure, or chemical composition, as opposed to reflecting the original *in vivo* condition.

Melzer's solution, and therefore chloral hydrate, is considered essential for evaluating a variety of amyloid and dextrinoid reactions. Chloral hydrate is a controlled substance and difficult to obtain engendering a search for a substitute. Visikol, a proprietary alcohol formulation with a high refractive index was evaluated by Leonard (26) and found to be unsuitable because it did not provide results similar to those of chloral hydrate. (Visikol contains a certain proportion of glycerol, which may negatively affect certain color reactions.) As previously noted, chloral hydrate's main benefit is in suppressing reactions considered to be non-specific (as well as homogenizing the cell content, reducing drifting, and delaying dehydration), and it is unlikely to find a substitute with exactly the same characteristics (26). Glycerol would be an obvious substitute since it can be mixed in various proportions to achieve specific refractive index values, but glycerol suppresses virtually all color reactions in the *Amanita* spores, while perhaps enhancing other color reactions (personal observations). Melzer's formulations, despite their numerous advantages are somewhat restrictive in terms of the fixed iodine concentration in the traditional formulas. Qualitative experiments with *Macrolepiota, Russula*, and spores from other species suggest that a lower concentration of iodine would often be preferable.

Chloral hydrate will gradually suppress the cytoplasmic staining in the *Amanita* spores, eventually leaving a blue or blue gray outline of the spore wall. With Lugol's, (and Melzer's as well) the cytoplasmic stain is reversible and can easily be washed out with water. In this regard, it is very easy to get a blue only reaction with aqueous iodine simply by rinsing. It is possible to visualize a dynamic "rainbow" effect (4) in very concentrated spore preparations by infiltrating iodine from the edge of the coverslip, and subsequently observing a reverse rainbow effect by washing out with water (results not shown). This indicates a relatively weak, reversible, although still positive reaction similar to those described by Baral in ascomycetes (3) where a certain titer of iodine is required to maintain a reaction in a state of dynamic equilibrium. The blue color reaction in the spore wall is relatively stable and the iodine complex will remain in place after washing out and even after drying. When dry, the blue complex becomes light red in *Amanita*, and intensely so in *Russula* (Fig. 6A and 6E). In contrast to this, the amyloid apical rings in the ascomycetes may remain blue upon drying and subsequent rehydration, suggesting a similar mechanism of iodine incorporation but different carbohydrate chemistries. The red staining in

the dry *Amanita* and *Russula* spores immediately becomes blue when rehydrated with water (Fig. 6C and 6F), but remains red when mounted in non-polar media (Fig. 6A, 6E) in this case immersion oil. The nature of the red complex is unknown and may consist of a potassium polyiodide salt but this is speculative. These salts are red in color but are unstable in water and immediately dissociate (11).

Popular literature states that the blue color of starch is due to triiodide which is partially true. Recent physical chemistry models suggest that it is a CT complex of either $I_2I_3^-I_2$ or $I_2I_5^-I_2$ iodine/triiodide that absorb red light and yield the blue color (42). The smallest complex of this type would consist of seven iodine atoms and with one atom per six starch monomers per iodine molecule, the carbohydrate chain would have a minimum length of 42 monomers, consistent with the 30 to 50 estimate.

The nature of the binding is of interest. It has been demonstrated through flow studies, that starch becomes more rigid with bound iodine, and that the iodine/iodine complexes are linear. The blue reactive iodine complex is not present in solution but is assembled and stabilized by the helical coils. In this sense, the starch behaves as part solvent, part scaffold that can assemble and stabilize novel molecules that do not exist under other conditions. It is possible that the hydrogen dense interior of the starch helix participates in the color reaction through this is still a matter of debate (16,42).

CONCLUSION

Amyloid *Amanita* spores develop differential color reactions in the spore wall and sporoplasm. The final observed color is the result of the combined colors from each compartment.
Inamyloid Amanita spores develop a weak, reversible color reaction in the sporoplasm only. This reaction is likely identical to the sporoplasm staining of the amyloid spores. Chloral hydrate inhibits the sporoplasm staining in both amyloid and inamyloid *Amanita* species.
Immediately upon staining in the case of amyloid spores, a distinctly green color reaction is observed in both Melzer's and Lugol's reagents. This color is consistent in the four sections of the Amanitaceae with amyloid spores (Lepidella, Amidella, Phalloidae and Validae).

Staining in the sporoplasm gradually fades with iodine stains containing chloral hydrate and the overall color of the spores shift from distinctly green to distinctly blue. The rate of the color change is dependent on temperature, iodine concentration, and overall spore concentration. Langeron's Melzer solution will develop a blue coloration more readily than the original 1924 Melzer solution due to the reduced iodine concentration in the modified formula. A blue only color reaction can be readily obtained with aqueous iodine either by washing out excess iodine with water, or drying and subsequently rehydrating spore preparations.

Chloral hydrate is considered essential, but is in fact not required to demonstrate the blue only amyloid reaction, and furthermore may mask important and possibly diagnostic color reactions similar to the hemiamyloid reaction seen in ascomycetes.

Chloral hydrate completely inhibits all dextrinoid type reactions in the hymenial layer of amyloid and inamyloid *Amanita* species. As a result, and considering that chloral hydrate is becoming increasingly difficult to obtain, perhaps it is time to consider phasing it out as an essential ingredient when evaluating iodine reactions in the white-spored Agaricales.

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