MICROCHEMICAL TESTS ON THE CELL WALLS OF CERTAIN FUNGI. CELLULOSE AND CHITIN

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The similarity of the life histories of the lower fungi and many of the lower algae would seem to warrant the expectation that the composition of their cell walls would have something in common. The analogies of structure and function observed in the Phycomycetes and the green algae indicate what is apparently a direct relationship between these forms. The Ascomycetes occupy a more doubtful position, appearing to be related to the red algae, or to the green algae through the intermediary of the Phycomycetes. The Basidiomycetes, however, show little or no relationship to either the lower fungi or to the algae. It would be expected that the Basidiomycetes would show a greater deviation from the algae in composition than would any other class of fungi.

Earlier workers believed that the cell walls of fungi were made up of a modification of cellulose which was called "fungocellulose." Richter (1881) made cellulose tests on *Agaricus campestris, Daedalea quercina, Polyporus fomentarius,* and Mucors. Chlor-zinc iodide and iodine-sulfuric acid tests were made on sections which had been previously treated with alkali. Positive cellulose reactions were obtained with all except the Mucors, which gave no certain result. The author concluded that fungocellulose was true cellulose.

De Bary (1887) was of a different opinion. The so-called "fungo-cellulose" differed from true cellulose in that it was insoluble in Schweitzer's reagent, and did not give the color with iodine which is characteristic of cellulose. True cellulose reactions, however, were given by the Saprolegnieae, *Protomyces macrosporus,* Peronosporeae, young Mucors, and the cells of the resting perithecium of *Penicillium glaucum.* Similar results are reported by Winterstein (1893) who removed fats and proteins from fungus

material, and found that the residue was insoluble in Schweitzer's reagent. He concluded that it was a cellulose differing from that in tissues of higher plants. Gilson (1893) was unable to obtain crystalline cellulose from *Mucor vulgaris, Thamnidium vulgare,* and *Agaricus campestris,* while he succeeded easily with plant tissue. He believed that fungus tissue did not contain cellulose.

Perhaps the greatest variety of materials was tested by van Wisselingh (1898). This author reports the presence of chitin in Myxomycetes, Peronosporales, Saprolegniales, Chitridiales, Entomophtharales, Mucolares, and in almost all of the higher fungi. Reactions for both chitin and cellulose were obtained with Myxomycetes and Phycomycetes, although the reactions were given by different portions of the hyphae. Chitin seemed to be confined to certain portions rather than to the entire cell wall. Wester (1909) prepared chitosan salts from *Mucor muceda, Xylaria hypoxylon, Peziza aurantia,* and *Xylaria polymorpha.* Chitin is reported by Vouk (1915) as being present in the following fungi: a Mucor, *Helvella crispa, Peziza aurantia, Xylaria polymorpha, Plicaria cervina, Agaricus fusipes, Amanitopsis plumbea, Boletus sanguinus,* a Clitocybe, *Cortinarius obtusus, Hygrophorus conicus, Mutinus caninus, Psalliota campestris,* and *Russula aeruginosa.* Extensive studies of chitosan salts were made by Brunswik (1921) These salts were prepared from *Lepiota procera, Pholiota squarrosa, Lycoperdon caulatum,* and an Aspergillus. These preparations were identical with chitosan salts of animal origin. Gwynne–Vaughan and Barnes (1927) made the statement that the cell wall of fungi is usually of cellulose, or of a special variety known as fungal cellulose.

Thomas (1928) using several species of Fusaria, extracted the proteins from the hyphae, and made an ammoniacal copper sulfate extraction of the residue. This reagent dissolved a material which appeared to be cellulose, giving cellulose color reactions, and hydrolyzing to reducing sugars. The hyphae were still intact, and gave tests for chitin.

The variations in results given by the above investigators may be attributed in part to the different methods used. One heated the material with glycerine to remove the cell

contents, while another let the sections stand in concentrated alkali to remove the protein constituents of the cells. Various adaptations of the tests for chitin and cellulose were used. With the many variable factors concerned, it is surprising that these workers did not report even more conflicting results.

The methods used in our work were those recommended by the most widely accepted authorities on micro-technique. Three tests for cellulose were employed, and one test for chitin.

Cellulose Tests.

1. Iodine-potassium iodide-sulfuric acid test. The sections were saturated with the iodine-potassium iodide reagent, the slide tilted, and the excess solution removed by placing a blotter at the lower edge of the drop. The blotter did not come in contact with the section, and any cellulose fibers adhering to the slide could not been confused with material in the section because of their difference in appearance. Cellulose gives a very deep blue color when treated with these reagents. Molisch (1923).

2. Iodine-potassium iodide-phosphoric acid test. This test was performed in the same way as the one above, except that it was necessary to heat the sections since the acid used was weaker than that in the first test, and consequently did not penetrate the cell wall as readily. Cellulose gives a deep violet with this treatment. Zimmermann (1901).

3. Chlor-zinc iodide test. This is one of the standard tests for cellulose, giving a dark violet-blue color with that compound. Molisch (1923).

Chitin Test.

Van Wisselingh (1898) sealed sections of material in glass tubes with concentrated potassium hydroxide solution. These sealed tubes were then heated in an oil bath to 180° C., cooled, the tubes broken, and the sections removed. The alkali was removed by washing with 90 per cent alcohol. After washing the section thoroughly with alcohol, it was placed on a slide, saturated with an iodine-potassium iodide reagent the excess of this solution removed as before

by blotting, and dilute sulfuric acid added. Chitin gives a violet color when treated in this way.

A much simpler method is given in detail by Vouk (1915) and recommended by Eckerson. This method consists in boiling the material for 20-30 minutes in a saturated aqueous solution of potassium hydroxide in a beaker and washing the material with 90 per cent alcohol. The remaining steps are not explicitly stated, but since this method was based on that of van Wisselingh, it is very probable that the remainder of the procedure was left unchanged, i. e., the sections were treated with iodine-potassium iodide reagent and sulfuric acid. Vouk first states that his preparations give the chitosan reaction "nach bekannten Weise mit Jodjodkalium." This statement does not exclude the use of sulfuric acid from the test. Further, this author states, "Nach dem Auswaschen in 90 p. ct. alkohol trat die Reaktion mit Jodjodkalium immer prompt ein." It seems doubtful that the use of sulfuric acid is indicated by this statement. Miss Eckerson, in adapting the method, does not state that sulfuric acid should be used. When the test as described by this author is applied to flies' bodies, no violet color appears, but it *does* appear when sulfuric acid is added. Other sources give tests for chitosan with iodine-potassium iodide reagents, and no sulfuric acid, but these tests all gave negative results when applied to parts of flies' bodies. The test for chitin was made by the procedure recommended by Vouk and Eckerson except that sulfuric acid was used to convert the chitosan to a material which gave a violet color with iodine-potassium iodide.

The number of materials to be tested for chitin made it impossible to boil them all together with alkali in a beaker. In order to keep them separated, and to easily distinguish the different materials tested, each was placed in an agglutination tube. These tubes were inserted for about half their length through the meshes of a screen of the proper size to hold them firmly. This screen, with the tubes held firmly in place, was then suspended in an oil bath. The samples in the tubes were covered with saturated KOH, the oil bath heated to 130° C, and this temperature was maintained for 25 minutes. The sections were then fished out

of the tubes, washed with 90 per cent alcohol, placed on a slide, and iodine-potassium iodide and sulfuric acid added.

Four cultures grown in the laboratory were tested daily, and tests were also made on material collected at random. The laboratory cultures were grown in Petri dishes as giant colonies on malt agar (3 per cent malt extract, 1.5 per cent agar.) Sections of the colony were removed daily, and the duplicate tests made upon it. The agar did not interfere materially with the tests, and in no case gave a color which would be mistaken for a positive test. The cultures grown on agar, and tested systematically were: *Mucor rouxii, Aspergillus fumigatus, Thraustotheca clavata,* and *Achlya imperfecta.*

In table 1 are given the results of the tests on *Mucor rouxii.* In the first 3 tests when the culture was 2, 4, and 5 days old respectively, all the tests were negative, although a pale violet color was given with chlor-zinc iodide, and on the last day a questionable chitin test was obtained. When the culture was 7 days old, colors which were very similar to these given by cellulose appeared with all of the cellulose test reagents, and a positive chitin test was obtained. On the 8th day, so much of the material had been removed from the first Petri dish that it was necessary to take material from another plate. This gave negative cellulose tests and a positive chitin test. Though both were started at the same time, their development may be very different, due to the vigor of the culture, the size of the inoculum, or a lack of uniformity of favorable conditions. The lack of concord between the results of different workers using the same fungus may well be due to such factors. On the 9th day, the chitin test was negative, while the cellulose tests were questionable, a color being obtained which was similar to that given by cellulose. On the 10th day, the cellulose tests, except the chlor-zinc iodide test, were positive, and the chitin test was negative. An old culture of *Mucor rouxii* which happened to be available was also tested. The cellulose tests except the chlor-zinc iodide test were positive, and the chitin test positive.

Aspergillus fumigatus gave somewhat different results. (Table 2.) At no time was a positive cellulose test obtained, although a pale violet color was observed with chlor-

zinc iodide from the 7th to the 10th day. The chitin test was questionable on the 4th and 5th day, but positive on the 7th and 8th day, negative on the 9th, and positive again on the 10th. On the 12th day, all tests were negative.

Thraustotheca clavata (table 3) and *Achlya imperfecta* (table 4) at all times gave identical tests. The cellulose tests were all strongly positive throughout, the color becoming very intense as the cultures increased in age. The chitin tests were all negative, although in the older cultures, a blue coloration appeared which, however, was quite different from the color given by chitin.

The results of the tests on the higher fungi are recorded in table 5. No form gave a positive cellulose test. Chitin tests were given by some, and not by others. In the cases where the chitin test was most positive, the violet coloration seemed to be confined to the cuticle, never extending far into the tissues.

Perhaps the greatest discrepancy occurs in table 1 in the cellulose tests. It is probable that the second culture used had not developed as far as the first one. In the chitin tests given in tables 1 and 2, there is a most discouraging lack of consistency. This may be due to several factors. The test in itself is not easily made. The sections used, though as small as possible, may nevertheless have been too large to enable the reagents to thoroughly penetrate the cell walls. The hyphae were always very much matted after boiling in alkali, and it was not possible to separate them. This made the test very difficult to observe, and may account for the lack of consistency of the results. The other two cultures used, *Thraustotheca clavata* and *Achlya imperfecta,* had such large and rigid hyphae that the tests were very easily observed.

In all of the tests made there is always a question regarding interfering substances. The cellulose tests were made in such a way that other substances giving a blue color with iodine would not be mistaken for cellulose. This was accomplished by saturating the section with iodine-potassium iodide reagent, and examining under the microscope before sulfuric acid was added. No blue color was observed in any case before addition of sulfuric acid.

Summary

Twenty-two species of fungi distributed through the Oomycetes, Zygomycetes, and Basidiomycetes, were tested for chitin and cellulose. The chitin test used was that of Vouk. Three cellulose tests were made: iodine-potassium iodide-sulfuric acid test, iodine-potassium iodide-phosphoric acid test, and the chlor-zinc iodide test.

Four fungi were grown on malt agar and tested daily. These were *Thraustotheca clavata, Achlya imperfecta, Mucor rouxii,* and *Aspergillus fumigatus.* The remaining fungi were tested as they were collected.

The lower fungi, Saprolegniales, gave strong tests for cellulose, one of the Mucorales gave tests for both cellulose and chitin at different ages, and the higher fungi gave negative tests for cellulose, though several gave chitin reactions.

It seems very likely that if these tests were made on a greater variety of forms, or systematic tests of cultured forms extended over a greater period of time, the results would permit more definite conclusions than are possible from this work.

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TABLE 1. *Cellulose and chitin tests on Mucor rouxii.*

Tests	Number of days							
	2	4	5	7	8	9	10	45
Cellulose.								
1. Iodine–potassium iodide–sulfuric acid	—	—	—	+ (?)	—	+ (?)	+	+
2. Iodine–potassium iodide–phosphoric acid	—	—	—	+ (?)	—	+ (?)	+	+
3. Chlor–zinc iodide	Pale violet	Pale violet	Pale violet	+ (?)	—	Pale violet	—	—
Chitin	—	—	+ (?)	+	+	—	—	+

TABLE 2. *Cellulose and chitin tests on Aspergillus fumigatus.*

Tests	Number of days						
	4	5	7	8	9	10	12
Cellulose.							
1. Iodine–potassium iodide–sulfuric acid	—	—	—	—	—	—	—
2. Iodine–potassium iodide—phosphoric acid	—	—	—	—	—	—	—
3. Chlor–zinc iodide	—	—	Very pale violet	Very pale violet	Very pale violet	Pale violet	—
Chitin	+ (?)	+ (?)	+	+	—	+	—

TABLE 3. *Cellulose and chitin tests on Thraustotheca clavata.*

Tests	Number of Days					
	4	5	7	8	9	10
Cellulose.						
1. Iodine–potassium iodide–sulfuric acid	+	+	++	++	++	++
2. Iodine–potassium iodide–phosphoric acid	+	+	++	++	++	++
3. Chlor–zinc iodide	+	+	++	++	++	++
Chitin	—	—	—	—	—	—

TABLE 4. *Cellulose and chitin tests on Achlya imperfecta.*

Tests	Number of Days					
	4	5	7	8	9	10
Cellulose.						
1. Iodine–potassium iodide–sulfuric acid	+	+	++	++	++	++
2. Iodine–potassium iodide–phosphoric acid	+	+	++	++	++	++
3. Chlor–zinc iodide	+	+	++	++	++	++
Chitin	—	—	—	—	—	—

TABLE 5. *Cellulose and chitin tests on higher fungi.*

Fungus tested	Cellulose. Iodine–potassium iodide sulfuric acid	Cellulose. Iodine–potassium iodide phosphoric acid	Cellulose. chlor–zinc iodide	Chitin
Xylaria polymorpha	—	—	—	—
Cordyceps militaris	—	—	—	—
Laboulbenia formicarium	—	—	—	+
Peziza bodia	—	—	—	—
Fomes applanatus	—	—	—	+
Fomes fomentarius	—	—	—	+
Polyporus betulinus	—	—	—	+
Polystictus hirsutus	—	—	—	+
Boletus felleus	—	—	—	—
Strobilomyces strobilaceous	—	—	—	—
Cantharellus cinnibarinus	—	—	—	—
Russula subdepallens	—	—	Slight violet on gills	+
Collybia platyphylla	—	—	—	+
Lactarius piperatus	—	—	—	—
Lactarius fuliginosus	—	—	—	—
Chamaeota sphaerospora	—	—	—	+ +
Pluteus nana	—	—	—	+ +
Amanita bisporiger	—	—	—	+