

UNCLASSIFIED

AD NUMBER
AD841608
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; JUL 1968. Other requests shall be referred to Commanding Officer, Fort Detrick, Attn: SMUFD-AE-T, Frederick, MD 21701.
AUTHORITY
Biological Defense Research Lab ltr dtd 13 Sep 1971

THIS PAGE IS UNCLASSIFIED

AD841608

TRANSLATION NO. 9

DATE: July 1968

DDC AVAILABILITY NOTICE

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of Commanding Officer, Fort Detrick, ATTN: SMUFD-AE-T, Frederick, Md. 21701.

OCT 24 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

PURE CHITIN PREPARATION FROM BOLETUS EDULIS, by Emil Scholl

(Presented at the 2 July 1908 meeting)

E. Winterstein, in a work on mushroom membranes, asks whether pure chitin may be produced from mushroom membranes, and on the basis of his research reaches the conclusion that it is not possible for the present. "The mushrooms certainly contain a chitin body; but it is always accompanied by carbohydrates, which in part are easily removed by diluted acids or alkalis, while others may only be diluted by concentrated sulfuric acid and may thereafter be hydrolyzed."

E. Gilson produced a body from agaricus campestris, which corresponds with chitin in its elementary construction, however he gives no data as to his preparation's reaction to concentrated alkalis, and does not deem it necessary to complete an elementary analysis during production of this substance from Boletus. He reaches the same results as E. Winterstein in the remainder of his work.

K. S. Iwanoff has occupied himself most recently with chitin production from mushrooms. By hydrolytic fission of membranes, obtained from Boletus edulis, with hydrochloric acid he only produced 40% chloride of glucosamin instead of 75 to 90% and reached the conclusion: "The analyses of mushroom cell membranes, in confirmation of the discoveries of E. Winterstein and E. Gilson, proved the presence of chitin, by which it would appear that chitin is connected with an unidentified substance rich in nitrogen."

Incidental to a study of animal chitin, which was conducted by O. V. Furth and by me, I observed that animal chitin showed reactions to concentrated alkalis quite different to those of the so-called mushroom cellulose. However both produce identical cleavage products: glucosamin and chitosan.

If one reads the rather extensive literature on animal chitin, one realizes that, despite the fact that very little is known about the constitution of this body, one is dealing with a substance of characteristic attributes. Furthermore chitin from the most varied sources is always insoluble in concentrated alkali solutions even after days of heating. The "mushroom cellulose preparates" produced by E. Winterstein react quite differently. "For the most part, they dissolved in cold, diluted, 5 to 10% lye". Chloride of glucosamine crystals, etc., were only obtained after dialysis by parchment tube after the hydrolytic fission with hydrochloric acid.

If one uses finely powdered, air dried mushrooms after extraction with alcohol, ether and water with 5 to 6% lye, despite which a small amount of solution penetrates, but contains no body which may be identified with chitin.

At present it is hard to accept that the preparates obtained by Winterstein basically resemble in their composition the construction of mushroom membranes, because Winterstein let acids and strong oxidation means, such as Schulze and Hopmeister mixtures, work into the mushrooms. Chitin stands out after this because of indifference to alkalis and light attackability by acids. These last qualities of chitin therefore point out the possible way of procuring chitin from mushrooms, when the mushroom membrane substance is completely identical with the chitin.

If one cooks the finely ground fruit bodies (stalks and heads) of *Boletus edulis* changing with water and 10% potash lye and eliminate the acid penetration which could cause hydrolysis, one may reach pure chitin, as shown below, in a very simple way.

Interesting in connection with this is the fact that, Braconnot in 1811, at a time when chitin was not yet known (it was first discovered by Odier in

1823 in insect wings and in the armor of crustaceans) arrived at a more or less white, soft, elastic, tasteless mass which he called Fungin, by pressing of fresh mushrooms and treating the residue with water, alcohol and alkalis. I am not in a position to judge how closely this fungin, which was produced in the same way as my chitin, actually resembled chitin, as the original work was not accessible.

Anyway the way opened by Braconnot was abandoned mainly on Payen's authority, and from then on almost all mushroom analysts followed the method below. The mushrooms were extracted with ether and alcohol and then treated with water, acids and alkalis, then followed an often week long cold penetration of Schulz and Hofmeister mixtures. Once Payen had had analysis results with his method, which was well adapted to cellulose, the method was followed until today, in studying cellulose presence and related carbohydrates and the efforts of all scientists before Gilson and Winterstein followed this line, in trying to discover the cellulose which was supposed but not found by normal reactions. Even De Bary who discovered the characteristic of mushroom membranes in that they did not show the characteristic iodine reaction of cellulose after treatment with potash lye and following digesting with potassium chlorate and nitric acid, just as they had been unsoluble in copperoxydammonia, and thought that it might at most be assumed that this was a new form of cellulose, which he called mushroom cellulose.

C. Richter working at the local institute upheld De Bary's mushroom cellulose theory in a work of great importance to my research. He let various mushrooms, among others Polyporus ribes and formentarius, soak in potash lye and then obtained violet and blue colorings resulting from chlorinated zinc iodide penetration. He deduced from this that mushroom membranes are composed

of cellulose. Gilson's and Winterstein's works only appeared a few years later, the assumption was included. In reality C. Richter must also have had chitin. Anyway both Winterstein's mushroom cellulose and the chitin I obtained from *Boletus edulis* produce blue or purple coloring when treated with chlorinated zinc iodide, however it is also possible, that chitin from the above mushrooms reacts to the said coloring, while animal chitin shows no basic reaction to iodine.

I feel that I have proof, as will be detailed below, contrary to the assertions of all those authors, that the *Boletus edulis* membranes are composed of pure chitin, which has the characteristics of animal chitin, at most very loosely united by a nitrogen-free carbohydrate and that this is probably true of all chitin containing mushrooms, which I shall attempt to prove in a later work.

II. Experimental Section

I used dried stumps and beads of *Boletus edulis* as starting material. The extremely clean mushrooms came from a dealer at Eisenstein in the Bohemian Forest.

The air dried mushrooms were pulverized and cooked with 20 parts water until the filtrate ran off nearly colorless. The mass which became shiny with cooling filtered very slowly through the pump. The process was accelerated by use of a hardened filter. The simplest process consists of carefully pipetting the liquid over the filter after a few hours of settling and blowing the substance deposited on the filter into a dish. The liquid is again placed on the cleaned filter and the process repeated until no liquid remains on the filter. Pollution of the substance with paper particles is completely impossible

when hardened filters are used. The cooking and filtering of 1000 g. of mushrooms in two siphon tubes 18.5 cm in diameter took a few weeks to complete. The use of 20 parts water is unconditionally recommended. Great complications arise during filtering if one employs less water.

After cooking with water a yellow-gray deposit remains, in which one may recognize the mushroom membranes under the microscope.

The mass produced in this manner was covered with the ten fold (1:10) mass of potash lye, and cooked for at least one hour. A dark brown color appeared nearly immediately after heating with strong ammonia evaporation. The reaction mixture was now very easily filtrable. The sediment was pressed as well as possible and was then cooked with water until the filtrate stopped being colored. I obtained a wine yellow filtrate after 3 to 4 heatings. The process, of repeated cooking with lye and water had to be repeated approximately four times. After the last cooking with potash lye the filtrate ran off wine yellow and gave no more reaction when subjected to acid. The substance was again washed with water and produced a yellow-gray, plastic, water logged mass. The pseudo-paranchyma could still be clearly recognized under a microscope. The damp mass appears yellow with chlorinated zinc iodide, brown with iodine potassium iodide and with iodine and sulfuric acid. The prepareate was quite as nonsoluble in the strongest solution of copper oxyammonia, as in concentrated potash lye under extreme heat.

For further purification a 1% potassium permanganate solution was added to the substance creating a thin paste and was allowed to remain in manganese peroxide until the potassium permanganate had completely penetrated. The brown mass was then pipetted off and heated with a highly diluted (1:40) solution of hydrochloride. The manganese peroxide dissolved in a few minutes and only the

nearly white mushroom membranes remained. They were repeatedly rinsed in water to eliminate the hydrochloride solution. The last acid remainders were extremely difficult to eliminate.

As the substance contained small resinous particles (acetic anhydride and sulfuric acid caused the known purple color), it was first heated with 96% alcohol and then another 3 times with 100% alcohol which was eliminated with water free ether.

After evaporation of the ether, the prepareate is dried in the air scarce chamber over sulfuric acid and later in the water drying closet until constancy of weight. By this method one may obtain 50g to 60g of sediment from 1000g of air dried mushrooms, or 5 to 6%.

The dried substance consists of pale gray crumbs, which are unsoluble even in prolonged cooking of potash lye. Sulfuric acid dissolves with strong brown color. The solution after dissolving in water strongly reduced Fehling's solution.

If the remains are placed from the 100% alcohol into the water bath, instead of processing through ether, the substance is obtained in more or less large, outwardly tenacious pieces, which could not be divided because of their toughness. Strikingly, the circumstance is here, that the sections against the glass plate are quite flat and red-brown colored, so that the appearance of the substance at least in these spots, reminds one strikingly of animal chitin.

The basic analysis of the body described above gave the following results.

I. Determination of carbon and hydrogen.

1. 0.3622 g of substance gave 0.2037 g H₂O

0.6012 g CO₂

0.0097 g ash = 2.68%

Therefore 0.3525 g of ash free substance produced

H	6.41%
C	45.51%

2. 0.3651 g of substance gave 0.2050 g H_2O

0.6007 g CO₂

0.0095 g ash = 2.60%

Therefore 0.3556 g of ash free substance produced

H	6.40%
C	46.07%

II. Nitrogen determination according to Kjeldahl.

1. 0.8634 g of substance, equaling 0.8406 g of ash free substance
used 35.9 cm³ 1/10 norm. H₂SO₄, i.e., 0.0526 g N = 5.98% N.

2. 0.7758 g of substance, or 0.7553 g of ash free substance, used
32.0 cm³ 1/10 norm. H₂SO₄, i.e., 0.0448 g N = 5.93% N.

3. 0.8525 g of substance, or 0.8300 g of ash free substance, used
35.8 cm³ 1/10 norm. H₂SO₄, i.e., 0.0501 g N = 6.04% N.

III. Volume nitrogen determination according to Dumas.

1. 0.227 g of substance or 0.221 g of ash free substance, produced 11.9 cm³ under 742 mm air pressure and 16° temperature, i.e., 0.01351 g N at 760 mm and 0° = 6.11% N.

2. 0.3255 g of substance, or 0.3169 g of ash free substance, produced 17.0 cm³ N under 742 mm air pressure and 16° temperature, i.e., 0.0193 g N at 760 mm and 0° = 6.08% N.

The results appear in the following manner:

Test No.	Carbon	Hydrogen	Nitrogen		Ash
	In Percentage				
1	46.51	6.41			2.68
2	46.07	6.40			2.60
1			5.98		
2			5.93		
3			6.04		
1				6.12	
2				6.08	

By comparing on the one hand the analysis results (in the next table) of E. Winterstein, K. S. Iwanoff and the numbers established by me and on the other hand the analysis numbers of animal chitin. The agreement between my results and those of animal chitin are very good, even though I have found less nitrogen than shown in later chitin analyses.

		C	H	N	ASH	
TYPE OF SUBSTANCE	AUTHOR	IN % IN THE ANALYSIS AVERAGE				REMARKS
Mushroom cellulose from Boletus edulis	E. Winterstein	43.49	6.37	3.61	4.55	
	K. S. Iwanoff	-	-	3.07	0.80	
Chitin from Boletus edulis	arrived at by my research	46.29	6.41	6.03	2.64	The nitrogen count from the average of 5 analyses
Animal Chitin	according to K. S. Iwanoff	46.21	6.28	8.22	-	
	according to O. v. Furth	46.37	6.48	6.42	-	average of 5 analyses

So as to assure myself of the purity of the obtained prepartate, I attempted to isolate certain shaped nitrogen free carbohydrates, respectively glycose such as osazone by the method established by E. Winterstein. The test proved the perfect purity of my substance.

I did the following:

10 g of substance were immersed in sulfuric acid and allowed to sit overnight. This was dissolved in a lot of water and cooked for approximately 5 hours. I ventralized with barium carbonate after cooling. The filtrate was pale yellow and strongly reduced Fehling's solution. After boiling down to syrup consistency it was pipetted twice with 96% alcohol. The collected filtrates were placed in a glass dish in the drier to be steamed. After steaming off of the alcohol both macroscopic and microscopic glycose crystals

could be seen. Attempts at producing a crystalized osazone by acetic acid and phenylhydrazin remained without results.

I feel that the following tests are another method of checking the accuracy of the result, that the preparate produced from *Boletus edulis* is actually pure chitin.

It is a known fact that one obtains as a side product the easily and nicely crystalizing glucosamin by hydrolysis of chitin and hydrochloric acid. Ledderhose produced it by cooking mashed lobster shells with concentrated hydrochloric acid and steaming this off until crystals form on the surface. He produced the hydrochloride of glucosamin in this manner with 70 to 75% yield.

E. Winterstein tried to follow the above method with his preperates from *Boletus edulis*. He obtained a dark colored liquid in which black, humic masses appeared. The filtrate, when steamed off produced no hydrochloride of glucosamin crystals. He was only able to obtain the crystalized secondary product, after he separated the filtrate from the black, humic masses of the dialysis by means of a parchment tube.

K. S. Iwanoff produced hydrochloride of glucosamin from *Boletus edulis* by the same method with approximately 40% yield.

The hydrolysis of my substance developed totally differently from that of E. Winterstein and K. S. Iwanoff.

After warming the substance covered with concentrated hydrochloric acid I obtained a dark brown solution, which was filtered after solution in water. A very limited dark brown deposit remained on the filter. The filtrate was concentrated and after settling all night, without dialysis, produced a large quantity of well formed, pale yellow crystals, which were easily soluble in

water and formed colorless crystals from this solution. The crystals are hard, have a bitter sweet taste and have reactions similar to those listed by E. Winterstein and K. S. Iwanoff.

The substance prepared by me from *Boletus edulis* is so pure that the above test may even be conducted microscopically.

A few hundredth's of a gram of the substance were covered with a few drops of hydrochloric acid and warmed over a water bath. Precipitation commenced. After careful concentration it was allowed to cool off and a small quantity of the deposit was placed on an object tray. By repeated enlargement one may see the beautiful and characteristic crystals of hydrochloride of glucosamin.

QUANTITATIVE CRYSTALLIZATION TEST

1. 9.74 g of substance, or 9.48 g of ash free substance, produced 7.1 g alcohol dried crystals of hydrochloride of glucosamin = 74.9%.

The mother liquor crystallization was lost by accident.

2. 10.8 g of substance or 10.51 g of ash free substance, produced 6.18 g of crystals after the first crystallization, after the second 2.04 g, totaling 8.22 g = 78.2%.

The mother liquor subjected to further steaming after the second crystallization produced no more crystals, but still strongly reduced Fehling's solution.

The body behavior is identical with melted potassium.

25 g of *Boletus edulis* chitin was placed in a flask with 100 g of potassium hydroxide and a little water and then heated in an oil bath for an hour at 180°. The pasty mass was diluted with water, nearly neutralized with sulfuric acid, the deposit was removed and washed off with water. I continued according to

3
Furth and Russo. The washed raw chitosan was diluted in weakened acetic acid and filtered a limited deposit remained on the filter. The filtrate was precipitated, well washed out, suspended in the least water possible, weakened hydrochloric acid added to the solution, and was then precipitated with concentrated hydrochloric acid. The precipitation was drawn off, diluted in a little hot water, and under intense heat mixed to enough concentrated hydrochloric acid, to keep the solution clear. It was then allowed to cool slowly.

This chitosan hydrochloride was again diluted and precipitated and the obtained pure chitosan was first washed with water until the disappearance of the alkali reaction, and the water was then removed with alcohol.

Dark brown crumbs appear after drying at 40° , which show the Lassaigne-nitrogen reaction. The hydrochloric solution does not reduce Fehling's solution and shows the property against nitric acid discovered by Furth and me.

If one mixes a watery hydrochloric chitosan solution, drop by drop, with a 5% sodium nitrate solution, a strong gas discharge results without nitric acid formation, and a body is obtained which is soluble in diluted acids, and in diluted alkalis but not in alcohol.

25 g of chitin from *Boletus edulis* produced 6.6 g of pure chitosan by the above method. Furth and Russo obtained 45 g of chitosan hydrochloride from 140 g of dried, cooled *Boletus* scales under propitious conditions. Taking the formula established by Furth and Russo, that 2 molecules of hydrochloric acid correspond to 1 molecule of chitosan, the 6.6 g chitosan obtained by me from 25 g of chitin would correspond to 7.7 g of chitosan hydrochloride, i.e., 30.8% while Furth and Russo obtained approximately 32%.

III. SUMMARY

1. It has been possible to produce pure chitin, from *Boletus edulis* (hoods and stumps) by penetration of 10% potash ley under great heat, without acids or strong means of oxidation. The yield corresponds to 5 or 6% of the weight of the air dried mushrooms.

2. The obtained chitin reacts exactly like animal chitin. In contradiction to substances prepared by Gilson, Winterstein and others it is quite nonsoluble in concentrated alkalis, while it is easily attacked by acids in hydrolysis.

3. The hydrolysis with hydrochloric acid follows the pattern described by Ladderhose in building glucosamin hydrochloride, therefore identical to animal chitin hydrolysis. A dialysis with hydrochloric acid after the heating as with mushroom cellulose is not necessary, for one immediately obtains well formed crystals of glucosamin hydrochloride from the concentrated solution. The yield is of approximately 78% crystals, while for instance K. S. Ivanoff obtained only about 40%.

4. As a further proof of the purity of the chitin produced by me, I must state that crystals of glucosamin hydrochloride from chitin from *Boletus edulis* were produced microchemically from a few hundredths of a gram of sand.

5. The chitin alkali melting parallels and, quantitatively follows, similarly as animal chitin.

6. The stand taken by Gilson, Winterstein, Iwanoff, etc. that chitin is joined by a nitrogen free carbohydrate does not apply to *Boletus edulis*.

The findings are very probably cleared up by the production method of "mushroom cellulose": The weekly acid penetration under cold, the Schulze

and Hofmeister mixtures. The carbohydrate formation must have taken place as a secondary reaction from the chitin.

7. The *Boletus edulis* membranes are mainly composed of pure chitin extremely loosely bound by nitrogen free carbohydrates. Seen from a chemical point of view this also applies microscopically. The pseudoparanchyme was still recognizable after the fourth cooking with potash lye.

8. It is suggested to drop the term "mushroom cellulose" in the sense applied by De Bary, so as to avoid generalities, and to speak of Fungin, in the sense given by Braconnot, in all cases where it has not been definitely established that the membranes are composed of chitin.

I am reserving the expansion of my method of research to various other mushrooms in the future.

Finally may I again express my deepest gratitude to my most respected teacher, Dr. Julius Wiesner, for his great advice and support.

Trans. fr. German
by Stancioff