CCXXXI. THE CHEMISTRY OF MOULD TISSUE.

II. THE RESISTANT CELL-WALL MATERIAL¹.

BY ARTHUR GEOFFREY NORMAN
AND WILLIAM HAROLD PETERSON.

*From the Departments of Agricultural Chemistry and Agricultural Bacteriology
of the University of Wisconsin, Madison, U.S.A.*

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THE cells walls of fungi lack the structure exhibited by those of higher plants, and at the same time are markedly inert towards the action of most reagents and stains. Many histological investigators, towards the close of the last century, occupied themselves with speculating on the nature of the cell-wall constituents. Tests for the usual structural constituents of plants were indefinite. Most of the workers assumed cellulose to be present, though so altered by combination or infiltration with other materials as to be difficult of characterisation. Several names were given to the cell-wall substance, the most general being "fungus cellulose." A chemical examination of this substance was made by Winterstein [1894; 1895] who showed it to be chitinous in nature. His investigations were mainly on material from higher fungi and his analysis showed about 3.6 to 3.9 % nitrogen in his preparations. From *Boletus edulis* Schöll [1908] obtained, by exhaustive extractions with hot 10 % KOH solution, a material which he described as pure chitin, yielding on hydrolysis 78 % of crystalline hexosamine hydrochloride. He concluded that the cell-walls of this organism are either composed of pure chitin or of chitin in very loose combination with a nitrogen-free carbohydrate. Later Sumi [1929] obtained chitin from the walls of spores of *Aspergillus oryzae*. He considered it to be present there in association with other substances. Proskuriakow [1926] has prepared from *Boletus*, *Agaricus* and *Polyporus*, by repeated extraction with 10 % NaOH followed by treatment with a solution of potassium permanganate, preparations of high nitrogen content (6.1–6.3 %). The actual amount of such material in the fungi is apparently small, since the yield from *Agaricus* was 5 % and from *Polyporus* 3–5 %. Takata [1929] by similar means obtained from *Aspergillus oryzae* 3.18 % of "chitin" containing 6.37 % N.

The purpose of these investigations was to examine the cell-wall material from *Aspergillus fischeri*, in comparison with that from higher fungi.

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EXPERIMENTAL.

Preparation.

After prolonged alkaline extraction of the tissue, a cream-coloured residue remained. This after careful washing and dehydration with alcohol and ether dried to a colourless mass and was readily powdered. If dried without alcoholic dehydration, or if this treatment was not exhaustive, the residue on drying became grey or brownish in colour, hard and vitreous in character and almost impossible to grind to a powder. The nitrogen content of this material varied according to the violence of the alkaline treatment employed, but was in general rather under 3.5 %. Some figures obtained for nitrogen content and the treatment employed are given below. These are not all however from the same batch of mould mycelium.

	% nitrogen	% ash
1. Boiled with 60 % NaOH for 1 hour	2.69	0.26
2. Boiled with 25 % KOH for 2 hours	2.78	—
3. Boiled twice with 10 % NaOH for 48 hours each time ...	3.10	0.59
4. Boiled with 5 % NaOH for 3 hours after pre-treatment with more dilute alkali	2.41	1.22
5. Boiled with 4 % NaOH for 1 hour	2.35	—
6. Boiled with 2 % NaOH for 3 hours	2.53	0.37

Tissue, which had been previously treated with a fat solvent, saponified with alcoholic NaOH and then exhaustively extracted with hot NaOH solution yielded about 20 % of this resistant fraction. When such a preparation was boiled for 2 or 3 hours with concentrated HCl, diluted and concentrated under reduced pressure to remove HCl, decolorised with norite and again concentrated to a small volume, crystals separated out which were identified as hexosamine hydrochloride. (N found, 6.45 %; theoretical, 6.49 %.) With phenylhydrazine the crystals gave glucosazone, and they titrated nearly quantitatively as glucose with the Shaffer-Hartmann alkaline copper reagent. (1.005 mg. hexosamine hydrochloride titrated as 0.805 mg. glucose; theoretical, 0.835 mg. glucose.)

The presence of hexosamine in the resistant fraction of the cell-wall of fungal mycelium has long been recognised and was, of course, responsible for its description as "chitin." Its identity with chitin from animal sources, however, must not be assumed. The nitrogen content of all these preparations was about 3 %; even after the most violent alkaline treatment, namely by boiling for 4 days with 10 % NaOH, it was not increased above 3.1 %. Chitin from animal sources contains over 6 %. The fungal preparations of Proskuriakow [1926] similarly contained over 6 % N. Since anhydrohexosamine ($C_6H_9O_4.NH_2$) has a nitrogen content of 8.7 % it is evident that the resistant complex in the case of *Aspergillus fischeri* is only in part composed of hexosamine units. A nitrogen content of 3.0 % would indicate only 34.5 % anhydrohexosamine. The nature of the remainder of this complex has not been determined. Some workers have referred to the presence of cellulose in

the cell walls of fungi, and Thomas [1928] claims to have demonstrated its presence in *Fusarium*. His data, however, seem hardly sufficient, since materials other than cellulose are soluble in ammoniacal copper solution. The evidence to be presented below indicates that cellulose, as ordinarily found in plants, is not present. Combination with hexosamine units, of course, might possibly so alter its character and properties as to make it unrecognisable, but even this is unlikely in view of certain results obtained on hydrolysis with dilute acids.

Properties.

This resistant residue was wholly insoluble in Schweitzer's cuprammonium solution and in a saturated solution of zinc chloride in HCl, both solvents for cellulose. When ground to pass a 100-mesh sieve it was, however, readily and completely soluble in 72 % H_2SO_4 in the cold giving a slightly brown liquid. A xanthate was obtained by stirring in CS_2 after treatment with 17 % NaOH solution in the cold. The xanthate had a typical golden-brown colour and gave a smooth thick paste. On dilution and acidification the original material separated out. It had, however, no tensile strength either as a film or as a fibre. This residue was carefully washed with water and dried with repeated changes of alcohol. The nitrogen content was found to be unchanged by the process.

Acetylation.

In order to demonstrate the identity of cellulose, should it be present in this fraction, several attempts were made at acetylation. 10 g. of tissue (N, 2.56 %) ground to pass a 100-mesh sieve were treated with 37.5 cc. of glacial acetic acid, 37.5 cc. of acetic anhydride and 1 cc. conc. H_2SO_4 as catalyst. After stirring for 2 minutes, the mass was allowed to stand for 30 minutes in a cold water-bath. The solution was then again stirred for 4.5 hours, diluted with 100 cc. glacial acetic acid and filtered. Very little appeared to have gone into solution. The clear filtrate was poured into 500 cc. distilled water and allowed to stand for several hours. A slightly milky emulsion was obtained on the addition of chloroform. The chloroform layer on separation was concentrated in the presence of a little water, upon which a granular white precipitate appeared in the water. This was centrifuged, washed several times in water and dried in a vacuum oven at 60°. The yield was very small, amounting to only 0.2 g. The acetyl content was determined by the method of Murray, Staud and Gray [1931] by solution in pyridine and treatment with NaOH at 53° for $\frac{1}{2}$ hour. (CH_3CO obtained 36.1 ± 1.5 %; theoretical for diacetylhexose anhydride, 34.9.) The residue was thoroughly washed and then suspended in water and extracted in a continuous extractor with isopropyl ether in order to remove all traces of free acetic acid. After drying with alcohol in a vacuum oven at 60°, the acetyl content was determined and found to be 5.8 %.

Acetylations were repeated under different conditions in an attempt to

obtain a product of higher acetyl content. By treatment of 10 g. of the resistant material with 0.5 g. ZnCl_2 , 40 cc. acetic anhydride and 40 cc. glacial acetic acid at 50° for 24 hours with continuous stirring, an insoluble product containing 6.02 % acetyl was obtained, but no soluble product as in the first experiment.

Barnett's [1921] method as modified by Irvine and Hirst [1922] with another sample gave an insoluble product containing 10.7 % acetyl, but only a trace of soluble product. This method involves the use of SOCl_2 as a catalyst, the temperature being maintained at 65° for 18 hours, and is the most effective method for acetylation of cellulose, which, under these conditions, goes readily into solution and gives the triacetate. Since no soluble product was obtained, the conclusion seems justified that cellulose in its normal condition was absent. No attempt was made to determine the position of the acetyl groups in the acetylated material. Even on the hypothetical basis of the formation of a mono-acetyl anhydrohexose or hexosamine, calculation shows that there has only been the introduction of one acetyl group for every two hexose or substituted hexose units in the molecule. This resistant structural material is clearly not easily acetylated.

Attempts were made to isolate substituted fission products by acetolysis. 10 g. tissue were treated with 40 cc. acetic anhydride and 5 cc. conc. H_2SO_4 in the cold, and allowed to stand at room temperature for prolonged periods. It was not found possible to isolate any product. In the cold the material failed to go into solution, and if warmed, charring and decomposition occurred.

Hydrolysis.

As ordinarily obtained, the ash of this material was very low, while the nitrogen present indicated from 33–40 % anhydrohexosamine. Hydrolysis can be effected with concentrated H_2SO_4 , but the recovery of reducing sugar expressed as glucose is far from complete. The hydrolyses were carried out as follows. About 0.5 g. of material passing a 100-mesh sieve was stirred with 10 cc. 80 % H_2SO_4 till completely dissolved, the beaker being kept cold during this process. After standing for varying periods in an ice-box, it was diluted to 200 cc. and the solution then autoclaved at 15 lb. for 1 hour. Reducing sugar was determined by the Shaffer-Hartmann method. Although the hydrolysis conditions were varied extensively, the highest yield of apparent sugar obtainable was 72 % calculated as anhydroglucose. The results obtained by this means were erratic, whereas good recovery can readily be obtained from cellulose. A small quantity of residue remained undissolved at the close of the hydrolysis. This was very difficult to determine accurately since washing was difficult and charring took place on drying. The amount varied from 5–10 %.

Acid hydrolysis under much milder conditions liberated appreciable quantities of reducing sugar. Table I indicates the percentage of apparent glucose from this resistant material on boiling with N and $N/2$ H_2SO_4 .

Table I. *Hydrolysis of resistant cell-wall material.*

Time in hours	Percentage apparent glucose liberated		Time in hours	Percentage apparent glucose liberated	
	$N H_2SO_4$	$N/2 H_2SO_4$		$N H_2SO_4$	$N/2 H_2SO_4$
$\frac{1}{2}$	8.6	2.8	15	33.5	—
1	8.9	4.7	23	38.8	—
3	15.9	10.9	28	—	30.1
4	20.3	14.0	32	—	30.7
6	25.7	15.6	39	51.1	—
8	—	19.6	48	—	37.2

The nitrogen liberated during the course of this hydrolysis was estimated only at the end of 24 hours and 39 hours with $N H_2SO_4$.

Table II. *N liberated on hydrolysis with $N H_2SO_4$.*

Time in hours	Total N %	N liberated from 100 g. g.	Calculated as hexosamine
24	2.77	1.44	18.4
39	2.77	1.75	22.4

The important fact which emerges from these results is that both the nitrogenous (chitinous) part and the non-nitrogenous part of this complex are hydrolysed slowly by dilute acid treatment. In order to investigate more fully the relative rates of hydrolysis, a further series of experiments was carried out under pressure and with a number of acid concentrations. By this means the charring which took place on long boiling with acid was avoided, except in two or three cases when the autoclave treatment was prolonged. A weighed amount of material (0.5 g.) was placed in a tube with 30 cc. of acid of the required strength, autoclaved at 14 lbs., and then made up to 50 cc. After being allowed to settle, aliquot portions were taken for the determination of apparent glucose, and soluble nitrogen, subsequently calculated as hexosamine. The residue was then filtered through a Gooch crucible, thoroughly washed, dried and weighed. The original resistant material contained N 3.1 % and ash 0.59 %. The results are presented in Fig. 1.

Fig. 1. Hydrolysis of resistant material with various strengths of H_2SO_4 at atmospheric pressure.

The evidence here presented indicates the very resistant nature of at least a portion of this residue; $N H_2SO_4$ at one atmosphere pressure removed 55 % in 4 hours and only an additional 8 % if the treatment was prolonged for a further 12 hours. Even then only 71.3 % of the nitrogen had been liberated. The original material on a basis of 3.1 % N contained 35.6 % anhydrohexosamine, while the residue at the end of 16 hours' treatment with $N H_2SO_4$

contained a very similar percentage, namely 34.7 % (1.12 g. N in 37.1 g. material). It should not, however, be deduced from this that the hydrolysis of the nitrogenous and non-nitrogenous units of this complex proceeded in parallel. This was definitely not the case for, in the early stages and under milder conditions, glucose or non-nitrogenous material was removed more rapidly than hexosamine. The approximate composition of the residue obtained throughout the 4-hour and 8-hour hydrolyses may similarly be calculated from the nitrogen not in solution. This is given in Table III. From this

Table III. *Approximate composition of hydrolysis residue.*

Concentration of acid	4 hours				8 hours			
	Residue %	Nitrogen in residue	Calculated as anhydro-hexosamine	Anhydro-hexosamine in residue %	Residue %	Nitrogen in residue	Calculated as anhydro-hexosamine	Anhydro-hexosamine in residue %
	45.1	1.98	22.7	50.3	—	—	—	—
0.8 N	46.2	2.0	23.0	49.9	41.5	1.72	19.8	47.7
0.6 N	52.0	2.13	24.5	47.2	43.0	1.86	21.4	49.8
0.4 N	57.1	2.30	26.4	46.2	47.8	2.13	24.5	51.3
0.2 N	60.7	2.44	27.1	44.7	58.5	2.20	25.3	43.3
0.1 N	63.5	2.67	30.7	48.4	62.3	2.44	28.0	45.0

it will be seen that practically one-half of the residue obtained in these two series was composed of anhydrohexosamine units. The remainder, however, cannot be considered as consisting solely of anhydroglucose units, though glucose was the only sugar detected as an osazone. The recovery throughout these hydrolysis experiments was far from complete and averaged about 80 %. By analogy with chitin from animal sources the presence of acidic groups was suspected, and this possibility was therefore examined. 1.362 g. of finely ground material were dissolved in 20 cc. 80 % H_2SO_4 , allowed to stand for 3 hours in an ice-box and diluted to 400 cc. The solution was autoclaved for 1 hour and distilled in steam, 4 litres of distillate being collected. The distillate titrated equivalent to 29.4 cc. 0.103 N NaOH. The volatile acid was characterised as acetic by the Virtanen-Duclaux determination. The residue from the distillation of volatile acid was extracted in a continuous extractor with isopropyl ether for non-volatile acid. The extract when taken up with water contained only a trace of acid. It would seem, therefore, that acetic acid is liberated on hydrolysis in appreciable amount and must be considered as a constituent of the molecule. The sample of resistant material used in the above experiment contained 0.37 % ash and 3.55 % N; CH_3CO content, 9.55 %. The anhydrohexosamine content ($\text{C}_6\text{H}_9\text{O}_4\cdot\text{NH}_2$) calculated from the nitrogen present was 40.8 %. Since, in the case of chitin from animal sources, the acetyl groups have been shown to be linked to the nitrogen of the glucosamine, it is not unlikely that a similar relationship may hold in this case. The acetyl content found corresponds very nearly to that required if there is one CH_3CO group for each hexosamine unit.

From this evidence obtained by hydrolysis it is not possible to make any very definite statement as to the nature of the resistant material. It is very improbable that a single entity is present. The changing ratio during hydrolysis between the hexosamine and the non-nitrogenous portion of the complex suggests the presence of two components hydrolysing at different rates. The fate of the acetyl groups in mild acid hydrolysis has not been determined, but the apparent production of a material containing about 50 % anhydro-hexosamine with widely different concentrations of acid is significant. These hydrolytic studies provide further evidence for the absence of cellulose, which, since it would be unaffected by the low concentrations of acid employed, would cause the ratio of the nitrogenous to the non-nitrogenous part of the complex to fall rather than to rise, as was observed. Tentatively it is suggested that this resistant cell-wall complex has two components. The major one consists of one molecule each of hexosamine, glucose and acetic acid, the last probably being linked to the nitrogen as in animal chitin. The other consists only of glucose units, is more readily hydrolysed and is soluble to some extent in strong alkali. Their association may be compared to that between xylan and cellulose in many plant fibres.

Fermentation of the resistant residue.

Still further to investigate the possible presence of cellulose in this fraction, portions of it were fermented with cellulose-decomposing organisms of high activity. 200 g. of wet residue (26.6 g. dry matter) were suspended in 500 cc. of a mineral salt solution and 5 g. of CaCO_3 added. After prolonged sterilisation, a small quantity of sterile peptone was added (0.5 %) and the whole shaken and inoculated with a heavy inoculum of the desired organism. Two fermentations were carried out (A) with a "purified" culture of thermophilic cellulose-decomposing organisms—very active in cellulose fermentation but containing several forms, mainly short rods, (B) with a purer culture containing the cellulose organism isolated by Snieszko (unpublished work) together with its two concomitant contaminants, which together bring about a rapid and extensive decomposition of pure cellulose.

Both fermentation mixtures were incubated at 55° for a period of 3 weeks. The fermentation was obviously very slight. At the conclusion of this period the volume was adjusted and aliquots taken for determination of the unfermented residue. These were washed with dilute HCl to remove carbonate and then thoroughly with water. The fermentations were also examined microscopically to verify the cultures employed. The loss of organic matter was found to be slight; in A, 10.72 % and in B, 8.76 %. While this cannot be taken as evidence for the absence of cellulose, it at least indicates that it is present only to a small extent, if at all, for under similar conditions pure cellulose would have been almost completely decomposed by these cultures in a much shorter period of time.

SUMMARY.

1. After exhaustive alkaline treatment of the tissue of *Aspergillus fischeri*, about 20 % of a resistant cell-wall material remained.
2. The preparations yielded hexosamine hydrochloride on hydrolysis with HCl and had a nitrogen content of about 3 %. This indicated the presence of approximately 35 % anhydrohexosamine in the complex.
3. The material was insoluble in cellulose solvents, but gave a xanthate.
4. Only a trace of a soluble acetylated derivative was obtained; the insoluble product of acetylation contained 10.7 % acetyl. Acetolysis resulted in charring and decomposition.
5. Hydrolysis could be effected readily after solution in concentrated H_2SO_4 , but the maximum recovery of apparent glucose was only 72 %. Dilute acid liberated both glucose and hexosamine slowly, and under pressure more rapidly. Glucose units were liberated more rapidly than hexosamine in the opening stages, and a residue containing about 50 % anhydrohexosamine was consistently obtained unless the treatment was violent.
6. Acetic acid was obtained on acid hydrolysis in amount approximately equivalent to one CH_3CO group for every hexosamine unit.
7. Fermentation with active cultures of thermophilic cellulose-decomposing organisms was slight, 10 % only being lost in 3 weeks.
8. The evidence suggests that cellulose, in the form usually met with in plants, is absent from this complex, which is probably a mixture of two components, one containing hexosamine, glucose and acetyl units, and the other, more readily hydrolysed, glucose units alone.

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