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Hydrolysis of the Chitinous Complex of Lower Fungi

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It has long been known that among the fungi certain constituents of the cell walls resistant to the action of strong alkali are not cellulosic, as in the higher plants, but are chitinous in nature. The question of the identity of plant and animal "chitin," which is still unsettled, has been reviewed recently by Rammelberg.[1] Comparatively little work has been done with the chitinous material derived from the lower fungi. The rather sparse literature has been reviewed briefly by Norman and Peterson,[2] who found that approximately 20% of the mycelium of *Aspergillus fischeri* was resistant to exhaustive alkaline treatment. This resistant material varied in nitrogen content, which in general was under 3.5%. Hydrolysis of the chitinous preparations with dilute sulfuric acid under pressure was incomplete, and the residue contained approximately 50% anhydroglucosamine, calculated from its nitrogen content. Norman and Peterson concluded that the alkali resistant fraction of the cell walls was probably a mixture of two components, one containing hexosamine, glucose and acetyl units and the other glucose units alone. However, Browne obtained a chitinous preparation from a *Citromyces* species which was apparently made up almost entirely of anhydroglucosamine units.[3]

In the course of an investigation on the nature of the ether soluble material of the mycelium of *Penicillium javanicum*,[4] it was found that a substantial fraction (up to 25%) of the dry, fat-free tissue was resistant to prolonged extraction with boiling 10% sodium hydroxide. This residue contained 4.0% nitrogen, which was equivalent to 46% anhydrohexosamine. Hydrolysis with concentrated hydrochloric acid (initially 37%) according to the procedure of Scholl[5] yielded crystals of glucosamine hydrochloride ($[\alpha]_D^{20}$ +72.5°), but in view of the nitrogen content of the sample the quantity recovered was much less than that expected. An appreciable quantity of humus-like material was formed during the hydrolysis.

(1) Rammelberg, *Botanisches Arch.*, **32**, 1 (1931).
(2) Norman and Peterson, *Biochem. J.*, **26**, 1946 (1932).
(3) Browne, THIS JOURNAL, **28**, 465 (1906).
(4) Ward and Jamieson, *ibid.*, **56**, 973 (1934).
(5) Scholl, *Monatsh.*, **29**, 1023 (1908).

Although hydrochloric acid has been used extensively for the hydrolysis of chitinous preparations derived from fungi, little attention has been given to its destructive action on the products of hydrolysis. To gain an idea of the extent of the formation of degradation products, some experiments have been carried out to ascertain the distribution of the nitrogen and reducing compounds in the hydrolysate.

Several preparations of chitinous complexes, which ranged in nitrogen content from 4.0 to 5.4%, were obtained from *P. javanicum*. Prolonged hydrolysis of a sample containing 4.34% nitrogen with concentrated hydrochloric acid (initially 37%) resulted in gradual solution of the preparation, but considerable insoluble humus eventually was formed. Nitrogen was determined in the filtered and decolorized hydrolysate, in the insoluble humus, in the glucosamine hydrochloride recovered, and in the final residual solution. The results of these analyses are assembled in Table I. Calculated from the reducing values,

TABLE I

DISTRIBUTION OF NITROGEN IN HYDROLYSATE OF CHITINOUS MATERIAL REFLUXED FOR THIRTEEN HOURS WITH CONCENTRATED HYDROCHLORIC ACID

Fraction	Wt., g.	N, mg.	% of N of sample	% of N of hydrolysate
Chitin sample	2.0555	89.4	100	..
Soluble hydrolysate	....	82.0	91.7	..
Humus	0.2683	6.3	7.0	..
Glucosamine hydrochloride	.6773	40.7	45.5	49.7
Residual solution	....	33.0	36.9	40.2

0.8220 g. of glucosamine hydrochloride was present in the hydrolysate, while 0.6773 g. of crude material was actually recovered. Calculated from the reducing value of the residual solution, only 0.073 g. of glucosamine hydrochloride was not recovered. From these results it is apparent that the large fraction of nitrogen remaining in the residual solution was not present as glucosamine hydrochloride, but represented either soluble non-reducing anhydrohexosamine, non-hexosamine compounds, or decomposition products of hexosamine.

Glucosamine hydrochloride is much more

stable than glucose when subjected to treatment identical with that given the chitinous material during hydrolysis. While the glucose was almost completely destroyed, as measured by copper reducing values, under the same conditions glucosamine was broken down to the extent of only 25%. It was also observed that the formation of humus was much less with glucosamine, since yields of only 0.4% by weight were obtained, while with glucose 15% yields resulted under the same conditions. The carbon and hydrogen content of humus arising from the hydrolysis of the chitinous complex was of the same order of magnitude as that of humus generally obtained by acid treatment of carbohydrates. It differed significantly, however, in that it contained 2.35% nitrogen.

If it is assumed that all the nitrogen of the chitinous complex is in the form of anhydrohexosamine, only 60% was present in the hydrolysate as glucosamine, as calculated from copper reducing values, and only 45.7% was recovered as crude glucosamine hydrochloride. Since control experiments indicated that only 25% of the glucosamine hydrochloride was destroyed under similar conditions, approximately 15% of the nitrogen remained, the origin of which could not be assigned to hexosamine on this basis. This nitrogen may be present in the chitinous complex in a non-hexosamine form, or may represent an anhydro-hexosamine fraction destroyed by hydrochloric acid prior to hydrolysis.

Experimental

The mycelia were grown from spores of *P. javanicum* introduced into a nutrient solution contained in aluminum pans enclosed in a specially designed cabinet, which will be described in a later publication. The composition of the nutrient medium is given below:

Substance	Gm./liter
Commercial glucose monohydrate	220.0
NH_4NO_3	2.25
KH_2PO_4	0.30
$MgSO_4 \cdot 7H_2O$	.25

After nineteen days' growth the mats were removed, broken up into small bits, and alternately squeezed and washed thoroughly with water. The tissue was dried at 90° for forty-eight hours, after which it was ground and the lipid material removed by continuous extraction with petroleum ether. The fat-free material was sieved, and that which passed 20 but which was retained by 40 mesh was reserved. This material contained 2.30% N; 100 g. of this tissue was boiled, with stirring, with 1 liter of 10% sodium hydroxide for ten hours. Appreciable quantities of an easily reduced pigment were extracted by the alkali. In the body of the suspension the color was a light yellow, while at the surface in contact with air it assumed an intense dusky purple shade. At the end of the extraction period, the suspension was filtered through linen, and the residue was boiled for ten hours with 10% sodium hydroxide. It was filtered, the residue stirred thoroughly with 300 cc. of water and again filtered. This operation was repeated four times. The residue was then stirred with 300 cc. of 0.2 *N* sulfuric acid, filtered and washed with two 300-cc. portions of water. The material at this stage was the color of cooked oatmeal. It was next suspended in 300 cc. of water, and 25 cc. of 6 *N* sulfuric acid and 10 cc. of 2% potassium permanganate were added with vigorous stirring. After filtration, the water suspension was cleared with sulfur dioxide and filtered, and the white residue washed with three 300-cc. portions of water, followed by a thorough boiling with 300 cc. of water. The washed residue was then stirred with 300 cc. of 10% ethanol, filtered, suspended in 25% ethanol for twenty-four hours, filtered and allowed to stand for twenty-four hours in 95% ethanol. After filtration it was dried at 75°; 17.44 g. of light brown material was obtained. Found: C, 45.04; H, 6.38; N, 4.34. Calculated for ($C_6H_9O_4NH_2$): C, 44.69; H, 6.88; N, 8.69. 67% of the nitrogen originally present in the mycelial tissue was removed by the alkaline treatment outlined above.

Hydrolysis of Chitinous Material.—2.0555 g. of chitinous material was hydrolyzed by refluxing with 100 cc. of concentrated hydrochloric acid (initially 37%) for six hours, at which time 50 cc. more of hydrochloric acid was added and refluxing continued for an additional seven hours. The solution gradually turned dark reddish-brown, and insoluble humus material separated out slowly. The brownish-black insoluble material was filtered off, washed with small quantities of water, and dried: weight, 0.2683 g.; C, 63.81; H, 6.76; N, 2.35. The filtrate was decolorized with char and made up to 500 cc. Aliquots were taken for determination of reducing value by the Schaffer–Hartmann method, and nitrogen by a semi-micro modified Kjeldahl method. The copper reducing value was equivalent to 0.8220 g. of glucosamine hydrochloride, which was equal to 29.9% anhydroglucosamine in the original sample. This calculation was based on copper reduction values found experimentally with pure glucosamine hydrochloride as follows.

Glucosamine hydrochloride, mg.	Copper, mg.	Glucose equivalent, mg.	Ratio of glucosamine hydrochloride to glucose
29.6	50.3	24.5	1.210
39.4	67.3	32.4	1.216
73.8	120.5	59.7	1.240
108.5	181.5	87.8	1.235
			Av. 1.225

The remainder of the hydrolysate (350 cc.) was evaporated under reduced pressure to a volume of 15 cc. and glucosamine hydrochloride precipitated at 0° with acetone. After standing for sixteen hours at 5°, the solid material was separated by filtration, washed with cold acetone, and dried at 90°: weight, 0.4641 g. or 0.6773 g. for the entire hydrolysate. Found: N, 6.01% (calculated for

$C_6H_{11}O_5NH_2.HCl$, 6.49%); $[\alpha]_D^{20}$ + 64.8° (pure $C_6H_{11}O_5NH_2Cl$ $[\alpha]_D^{20}$ + 72.5°). The filtrate from the crude glucosamine hydrochloride was diluted with water and the acetone removed by repeated dilution with water and distillation. The residual solution was then analyzed for nitrogen and reducing compounds: found, 33.0 mg. N; copper reducing value equivalent to 73.0 mg. of glucosamine hydrochloride.

Comparative Stability of Glucose and Glucosamine Hydrochloride toward Boiling Hydrochloric Acid.—0.6941 g. of glucose monohydrate (0.6316 g. anhydrous glucose) was refluxed with 50 cc. of concentrated hydrochloric acid (initially 37%). After a few minutes of boiling, the solution turned dark brown, and insoluble material began to separate out. After refluxing for ten hours, the solution was filtered, and the humus was washed with a small quantity of water and dried at 100°: weight, 0.0961 g., equivalent to 15.2% yield by weight. The copper reducing value of the filtrate was equivalent to 24.9 mg. glucose, equal to 3.9% of the sample, indicating that 96.1% was destroyed.

0.6241 g. of pure glucosamine hydrochloride was subjected to the same treatment as the glucose sample; weight of humus material, 0.0027 g., equivalent to 0.4% yield by weight. The copper reducing value of the filtrate was equivalent to 0.4710 g. of glucosamine hydrochloride, equal to 75.4% of the sample, indicating that 24.6% was destroyed.

Summary

A chitinous complex was isolated from the mycelium of *Penicillium javanicum* in quantity equivalent to 17.4% of the fat-free tissue, which contained 45.04% carbon, 6.38% hydrogen and 4.34% nitrogen. Hydrolysis of this material with hydrochloric acid of an initial concentration of 37% resulted in considerable destruction of the primary products of hydrolysis. The nitrogen of the hydrolyzed chitinous complex was distributed as follows: 7.0% in insoluble humus-like material; 45.5% in glucosamine hydrochloride; and 36.9% in non-reducing nitrogen compounds remaining in solution after recovery of the glucosamine. Insoluble nitrogen-containing humus-like material was formed to the extent of 13.0% by weight, probably chiefly from glucose anhydride units in the chitinous complex. Glucosamine hydrochloride was decomposed to the extent of 25% by prolonged boiling with concentrated hydrochloric acid, with the formation of 0.4% by weight of humus-like material.

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Rate of Carbohydrate Condensation on a Cell Wall

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It is possible to determine the rate at which cell-wall material is deposited during the growth of a new cross wall in certain green algae. Observations of that process were made on dividing cells of a species of *Rhizoclonium*, a genus in the family *Cladophoraceae*, in which the cross wall grows from the side walls toward the center of the cell. The alga is a filament consisting of a single row of cylindrical cells placed end to end. The cells are about 100 microns long and 20 microns in diameter. The new cross wall starts as a ring-like projection on the inside of the wall and grows by addition of material on its inner edge, gradually building the ring into a solid disk, somewhat as an iris diaphragm shutter of a camera may be slowly closed to form a solid disk. When the disk is completed, the cell is divided into two new cells, equal in size.

The growth of the new wall was observed under the microscope and measurements were made of the progress of growth. An eyepiece micrometer was used for making the measurements. Its smallest unit was equivalent to 4 microns (0.004 mm.) when the magnification was 440 diameters. Estimations to 1 micron were therefore fairly accurate.

The green cylindrical threads when seen under the microscope appeared as two-dimensional flat threads consisting of rectangular cells, end to end; and the growing cross walls were seen as minute projections extending inward from the side walls as indicated in the figure. The growth of the cross walls, as seen on this edge view, was indicated by the slow increase made in the length of these lateral projections. The growth continued until the lines met, closing the wall completely across the cell. The new cross wall thus formed was a disk 20 microns in diameter and 1 micron in thickness. There was no change in thickness from the time it started as a minute projection on the side wall of the cell until it had grown into the completed solid disk.