XXIX. THE ORIGIN OF THE GLUCOSAMINE OBTAINED IN THE HYDROLYSIS OF *BOLETUS EDULIS*.

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The existence of chitin in fungus cellulose has long been known, but the question of the origin of the glucosamine obtained in the hydrolysis of dried fungus extract has given rise to two distinct views. The first view is that the glucosamine residue is pre-existent, either as glucoproteins, or as glucosamine glucosides. It has been proved by Müller [1901] that glucoproteins give on hydrolysis a carbohydrate compound identical with that obtained from chitin. The second theory is, that glucosamine is an adventitious product, formed in the process of hydrolysis by the interaction of carbohydrate residues and amino acids.

From the results of numerous experiments, strong evidence in favour of the first theory has been obtained. In particular, reference must be made to the recent isolation of definite glucosamine glucosides by Yotake and Sera [1912]. The material used in their investigations was a species of *Lycoperdon*, (*L. gemmatum*, *Batsch*), and, by hydrolysis with concentrated mineral acid, they obtained a compound, termed by them "lycoperdin," which from its reactions proved to be an amino glucoside containing two glucosamine residues. Still more recently, they obtained the same compound from other species of *Lycoperdon* (*L. bovista*), and also from *Geaster granulatus*.

With the object of isolating similar glucosides, the following research was carried out on *Boletus edulis*. This particular fungus was selected, as it was already known that a large proportion of fungus cellulose existed in the plant, and, therefore, it seemed a probable source of amino glucosides.

Investigations on *Boletus edulis* had previously been carried out by Reuter [1912], who examined various extracts, but more particularly those yielding amino acids. He finally obtained a fraction containing the carbohydrate residues, but did not examine it in detail. It was accordingly decided to examine this carbohydrate fraction for the presence of amino glucosides.

In the extraction of the fungus material, Reuter's experimental method was carefully followed, and thereafter the examination of the dried extract was carried out according to the process of Yotake and Sera [1912]. Hydrolysis was effected by sulphuric acid, and phosphotungstic acid used to precipitate amino compounds. Owing to a slight solubility of certain of these compounds in phosphotungstic acid, both precipitate and filtrate were examined. Three amorphous products were obtained (as described in the experimental part). These were optically active and contained both carbohydrate and amino groups. From the filtrate a compound was isolated which showed reducing properties, but on hydrolysis the power of reduction practically disappeared. The remaining two products showed no sign of reduction either before or after hydrolysis. The examination thus gave no trace of glucosamine glucosides or of complexes containing glucosamine. The substances actually isolated appear to be condensation compounds of sugars with amino acids.

The absence of glucosamine from these compounds indicates that in *Boletus edulis* the amino sugar does not occur in the form of an amino glucoside, as otherwise glucosamine would necessarily have been obtained by these methods.

There remained, however, the possibility that the extraction of the carbohydrate fraction of *Boletus edulis* by means of sulphuric acid had been incomplete, and accordingly the undissolved residue was further examined. It was treated with concentrated hydrochloric acid, and kept just below boiling point for seven or eight hours. The insoluble material was filtered, and the solution concentrated and examined for glucosamine. Again no trace of this compound was found.

As however Reuter [1912] had obtained a definite amount of glucosamine from the carbohydrate fraction of *Boletus*, another hydrolysis was carried out on the original material, using on this occasion concentrated hydrochloric acid. The result confirmed Reuter's experience, as glucosamine hydrochloride was isolated in amount practically identical with that obtained by him.

These observations seem on first inspection contradictory, but they indicate that the production of glucosamine from *Boletus edulis* must be due to the formation of the amino sugar during the process of hydrolysis with concentrated hydrochloric acid. Hydrolysis with sulphuric acid yields a solution and a residue, neither of which contains glucosamine compounds. Since it is known that the action of concentrated sulphuric acid on chitin gives various results, in some cases entirely removing the amino group as ammonia, the absence of glucosamine in the products isolated might be accounted for in that way.

The work of Yotake and Sera [1912] however makes this seem improbable, as they isolated amino glucosides from reactions in which concentrated sulphuric acid was used. The conclusion arrived at therefore, is, that the glucosamine isolated from *Boletus edulis* under certain conditions is derived from a complex, of the nature of a glucoprotein, containing carbohydrate and amino acid residues, but does not exist in the form of a glucosamine glucoside.

This idea may be formulated as follows:

where (I) represents one characteristic grouping in a glucoprotein, which hydrolyses in two alternative ways, according to the hydrolyst used, giving rise to cleavage products (II) (a sugar and an acid amide); or to the products (III) (an amino sugar and an acid). Subsequent hydrolytic action on products (II) would yield ammonia and a carboxylic acid.

EXPERIMENTAL.

The *Boletus edulis* was supplied specially for the purpose of this research by Messrs Caesar and Loretz, of Halle, and the extraction of the material was carried out by Burroughs, Wellcome & Co., according to Reuter's method [1912]. The total weight received by them was 3565 grams, but after drying and grinding this was reduced to 3266 grams. The different extracts were:

(a) Ether giving	..	..	..	72.3 grams
(b) Alcohol ,,	..	..	..	1061.2 grams
(c) Water ,,	..	..	..	785.3 grams

From aqueous washings 71.5 grams were obtained. The remaining undissolved matter weighed 1040 grams.

The material used in the research was the dried powdered residue left after extraction with water. As the method employed involved the manipulation of solutions of very large bulk, experiments were carried out on the scale described below, and since the products isolated were not fully examined and therefore cannot be definitely named, a system of lettering has been adopted to avoid confusion.

70 grams of the dried residue were ground in a mortar with 70 cc. of concentrated sulphuric acid. A thick paste was formed to which 350 cc. of water were added. The mixture was transferred to a large flask and boiled under a reflux condenser for six hours. The deep brown solution was filtered from the undissolved residue containing humic acid salts; the latter was again extracted with dilute sulphuric acid, boiled for some time, and the liquid filtered, leaving the undissolved *Residue A*.

The joint filtrates were then partially neutralised by the addition of a cold saturated solution of barium hydroxide, in order to remove 95 per cent. of the sulphuric acid present. The precipitate was filtered and the brown filtrate decolourised by boiling with charcoal. After being allowed to stand for forty-eight hours the solution was again filtered and 10 per cent. phosphotungstic acid solution added until precipitation was complete. From this, *Precipitate B* and *Filtrate B* were obtained. Before precipitation with phosphotungstic acid, polarimetric examination of the clear solution showed that it possessed a slight but perceptible dextrorotation, and, after precipitation, *Filtrate B* still showed optical activity.

Examination of Precipitate B. The light brown precipitate of phosphotungstates was washed by continued decantation with 5 per cent. sulphuric acid, filtered, rubbed with dilute sulphuric acid and again filtered. Finally, it was dissolved in dilute acid, leaving a dark residue (*Residue D*) which was filtered, and the clear solution was then treated with barium hydroxide until only a slight acidity remained. Barium sulphate was removed by filtering, the solution was saturated with carbon dioxide, and again filtered. In order to remove the last traces of barium, dilute sulphuric acid was cautiously added until a very slight acid reaction was produced. The clear solution was concentrated on the water bath at 30°–40° under diminished pressure, and when reduced to very small bulk, was further concentrated in vacuum until a thick syrup remained. On the addition of absolute alcohol, a white

precipitate was obtained (*Precipitate C*), which was filtered, washed with absolute alcohol, and dried.

Examination of Precipitate C. The product obtained was an amorphous yellow powder, deliquescent, insoluble in alcohol and ether. The melting point was somewhat indefinite (46° – 50°), and the compound showed no action on Fehling's solution either before or after hydrolysis. Nitrogen was present in the form of amino nitrogen as indicated by the reaction with nitrous acid. Traces of carbohydrate were also present as shown by Molisch's test. In aqueous solution the compound showed a very slight rotation, $[\alpha]_D = +4.1^{\circ}$. As it was evident that this product contained no glucosamine residue, it was not further examined.

Examination of Residue D. The dark-brown undissolved material from recrystallisation of the phosphotungstic acid precipitate was dissolved in water, and treated in the same way as *Precipitate C*. It was similarly decomposed by barium hydroxide and, after filtering, the highly coloured filtrate was saturated with carbon dioxide, made slightly acid with dilute sulphuric acid, and concentrated in vacuum to a thick syrup. Precipitation with absolute alcohol gave a dark brown, amorphous, deliquescent powder. The product contained amino nitrogen, but showed no sign of reduction with Fehling's solution either before or after hydrolysis with concentrated hydrochloric acid, so that glucosamine complexes were absent. It gave no biuret reaction.

Examination of Filtrate B. The phosphotungstic acid filtrate was neutralised with barium hydroxide, the precipitate filtered and the solution concentrated on the water bath at 40° and finally in vacuum until a thick syrup was obtained. From this, on precipitation with absolute alcohol, a white product separated, which was filtered, washed with absolute alcohol and dried (*Precipitate E*).

Examination of Precipitate E. The substance was a white amorphous powder, soluble in water, insoluble in alcohol and ether. Ignition on platinum gave an inorganic residue consisting of barium salts. The compound contained amino nitrogen, readily reduced Fehling's solution, and in aqueous solution showed a slight rotation. Unsuccessful attempts were made to remove the inorganic matter. Part of it was precipitated as barium sulphate, but even after further purification barium salts still remained. The percentage of barium was therefore estimated and the product analysed.

Melting point 78° – 80° (indefinite). The substance darkened slightly, and began to char at 110° .

Analysis of Purified Precipitate E.

Barium	12.43 %
Carbon	37.84 %
Hydrogen	6.97 %
Amino Nitrogen	2.325 %
Total Nitrogen	2.719 %
Rotation (in water) $[\alpha]_D$	- 13.3°

Hydrolysis with concentrated hydrochloric acid gave a yellow crystalline product which showed no reducing properties.

The high percentage of barium indicates that the compound is a salt, moreover the action on Fehling's solution observed before hydrolysis indicates that a carbohydrate group is present, and the fact that the total nitrogen is combined as amino nitrogen is important. It would thus appear that the compound is the barium salt of a carbohydrate acid combined with amino residues. These amino groups cannot however be attached to the penultimate position in the sugar chain, as otherwise glucosamine would have been produced on complete hydrolysis. This has already been shown from the results obtained by Irvine, Thomson and Garrett [1913], and by Fischer [1902].

Examination of undissolved Residue A. Since none of the products isolated showed any trace of the presence of glucosamine, *Residue A* was examined. It was shaken with concentrated hydrochloric acid for twelve hours, and then boiled gently under a reflux condenser for six hours. The undissolved material was filtered, and the dark-brown solution concentrated on the water bath under diminished pressure. No crystals of glucosamine hydrochloride separated, and precipitation with alcohol gave a slight dark-brown precipitate which showed no sign of reduction with Fehling's solution.

The barium sulphate precipitate obtained before precipitation with phosphotungstic acid was similarly extracted, and gave the same result.

As this process is the standard method for preparing glucosamine, it is evident that derivatives of the amino sugar could not be present.

Hydrolysis of the Original Extract with Concentrated Hydrochloric Acid.

50 grams of the original extract were treated with 600 cc. of concentrated hydrochloric acid, and boiled under a condenser for seven hours. The solution was diluted and filtered, and then concentrated on the water bath at 50°. A crystalline product separated, which was filtered, dried, and recrystallised

from water. It readily reduced Fehling's solution, and contained amino nitrogen.

Permanent rotation of the compound in water, $[\alpha]_D = +71.5^\circ$.

The amino nitrogen was estimated and found to be 7.567 %.

Glucosamine hydrochloride requires $[\alpha]_D = +72.5^\circ$; Amino nitrogen = 7.86 %.

The substance was undoubtedly glucosamine hydrochloride. The total yield of the recrystallised product was 1.7 grams which corresponds with the amount obtained by Reuter [1912] from a similar process of hydrolysis.

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