XXXII. THE STEROLS AND CARBOHYDRATES IN FUNGI

I. *BOLETUS EDULIS*

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(Received 17 December 1936)

STEROLS are very widely distributed in the vegetable kingdom and have been isolated from the seeds, roots and rhizomes, stems, leaves and flowers of a great variety of plants, from vegetable oils and milk sap of certain plants, in which they occur partly in the free state and partly combined in the form of esters and other combinations.

Fungi appear to differ both quantitatively and qualitatively in their sterol content, e.g. certain bacteria contain no sterols.

The true fungi contain a certain peculiar group of sterols which is not present in Phanerogams. The fungus sterols differ in a characteristic manner from the cholesterol and sitosterol groups in their behaviour when shaken in chloroform solution with strong sulphuric acid. In the case of the fungus sterols, the acid becomes coloured a deep red and the chloroform remains quite colourless, whereas with cholesterol or sitosterol the chloroform is coloured red and the sulphuric acid shows a green fluorescence. This reversed Salkowski test reaction of the fungus sterols, however, is also given by some cholesterol derivatives such as cholesterol oxide and cholestenone. The fungus sterols give the Burchard-Liebermann test and also form sparingly soluble compounds with digitonin.

Tanret [1889] isolated a sterol from spurred rye, and subsequently [1908] found that this sterol contained two sterols, ergosterol and fungisterol, which he fully described. He prepared a number of esters and investigated their properties.

The presence of ergosterol in yeast was first noticed by Gérard [1895]. The recent discovery, however, of the close relationship of vitamins to sterols led to extensive investigations of the sterols of yeast by various workers, notably Smedley-MacLean & Thomas [1920].

It has been known for some time that many members of the fungus group contain mixtures of sterols and Mr J. A. Gardner thought that it might prove interesting to examine some of the commonly occurring fungi more thoroughly.

The first fungus studied was *Boletus edulis*, which is utilized as food in Russia and comes on the market in a dried state.

The other fungi under investigation at the moment are *Polyporus squamosus* and *Coprinus atramentarius*.

EXPERIMENTAL

In preliminary experiments the extracting powers of boiling alcohol, ether, chloroform and benzene were tested and it was found that the completeness of extraction depended more on the fineness of division of the material than on the solvent. Eventually ether was chosen as the solvent and the bulk of the sterol was extracted by allowing the finely ground powder to stand for a considerable

time in darkness in contact with cold ether. After filtering off the ethereal solution, the grounds were repeatedly extracted with boiling ether under a reflux. It was not found possible to obtain an extract which was absolutely sterol-free however long the extraction was continued. Extraction in a Soxhlet apparatus was not found more effective than the above method. Hydrolysis of the extracted grounds with 5% alcoholic potash, in order to break down any possible compound of the sterols with protein or other substance, subsequent dilution of the fluid and extraction with ether yielded only traces of sterol.

The ethereal extracts were dried with anhydrous Na_2SO_4 , the ether distilled to a small bulk and the crystals, which separated out, fractionally recrystallized from ethyl alcohol and eventually from a mixture of benzene and absolute alcohol (1:3). Later experiments showed that it was better to crystallize from a mixture of benzene and alcohol straight away.

The purified product melted between 164 and 165°. The rotation was observed on a sample dried in the steam oven. In a chloroform solution $[\alpha]_D^{18} = -125.46^\circ$. The following derivatives were prepared.

The *acetate* was prepared in the usual way by boiling with acetic anhydride in a matrass. The yield was satisfactory. After several recrystallizations from a mixture of 3 parts of acetone and 1 part of ether, the acetate had a melting-point of 173° and a specific rotation in chloroform -79.538° . This value is somewhat lower than that found by Preuss *et al.* [1932], and Reindel *et al.* [1927].

A *urethane* was made from a sample of slightly impure ergosterol melting between 153–158°. 2 g. of this were mixed with 2 ml. phenyl isocyanate and the tube placed in a paraffin bath at 178–183° for 4 min. The crude product was crystallized from ethyl acetate. The crystalline matter so obtained was treated with chloroform to separate the urethane from diphenylurea obtained as a by-product and the chloroform solution, after filtering and evaporating to dryness, was recrystallized twice from ethyl acetate, then once from ethyl alcohol and twice more from ethyl acetate, when crystals melting at 183° were obtained.

Analysis of the urethane.

Found: C, 81.281; H, 9.559; N, 2.932%. $\text{C}_{24}\text{H}_{44}\text{O}_2\text{N}$ requires: C, 81.44; H, 9.38; N, 2.79%.

The diphenylurea which was found as a by-product was identified by its melting-point and analysis.

Hydrolysis of urethane. The urethane was hydrolysed by boiling under reflux for 2 hours with 10% alcoholic potash. The thick crystalline mass obtained on cooling and standing was filtered off and dissolved in ether. The ethereal solution, freed from alkali by washing, was evaporated to dryness and fractionally recrystallized from a mixture of ether and ethyl acetate; it melted at 161°. From the mother-liquor crops with m.p. varying from 145 to 153° were also obtained.

Ergosterol obtained from this mushroom, although similar in its general properties and constants to ergosterol obtained from yeast, which is found on the market commercially, seems to be more stable in character. On keeping, the melting-point and the colour remain unchanged for a long time, unlike the changes in yeast sterol noticed by many observers.

Spinasterol. The original mother-liquors from which all the ergosterol had been crystallized were found to contain mainly fatty acids, which, however, still showed the Burchard-Liebermann reaction. They were neutralized with NaOH and evaporated to dryness. The dry residue was extracted by boiling with small portions of ether. The residue obtained by distilling the ether still weighed 8 g.; it was unidentifiable and still gave a positive Burchard-Liebermann

test. In the hope of isolating a sterol from this as a urethane, it was dissolved in benzene, dried with CaCl_2 and after filtering was boiled under a reflux with 4 ml. of phenyl isocyanate for 2 hours. The bulk of the material which separated on standing was a solid, which was filtered off. All attempts failed to obtain it in a pure crystalline form for identification, but the mother-liquors on evaporation were divided into chloroform-soluble and chloroform-insoluble portions. The insoluble portion was found to be diphenylurea.

The non-crystallizable solid material and the chloroform-soluble mother-liquors were hydrolysed by boiling under a reflux with alcoholic potash. The alkaline liquors were extracted with ether and on concentration a small quantity of a crystalline solid separated out, which was filtered off.

This solid was heated with benzene, but only a small portion dissolved. The insoluble part proved to be diphenylurea. The benzene solution and the ethereal mother-liquors were evaporated to dryness and recrystallized once from ethyl alcohol by adding water to faint turbidity. The crystalline product so obtained became opaque on heating at 164° and ran in the tube at 169° . From its appearance and behaviour it was not ergosterol and recalled the properties of spinasterol.

The acetate prepared in the usual manner melted at $174\text{--}175^\circ$. The total amount was only 0.002 g. The acetate gave a Burchard-Liebermann positive reaction and the Rosenheim test was negative. Probably the mushrooms contain a small quantity of spinasterol.

Carbohydrates. It is stated that "trehalose replaces sucrose in those fungi which do not contain chlorophyll and do not manufacture starch. The quantity of trehalose is a maximum just before the formation of spores: when the fungi are picked, the trehalose is rapidly converted into mannitol, being hydrolysed by trehalase to glucose within 2 or 3 hours after gathering" [Armstrong & Armstrong, 1934]. If this is true, the process must have been stopped in *Boletus edulis* by drying, since we found large quantities of trehalose, but were unable to isolate any mannitol, though this has been stated to be present by Margewicz [1883].

We may mention, however, that on extraction of *Armeleria mellea* we easily isolated considerable quantities of mannitol, but no trehalose.

Extraction. The dried and finely powdered mushrooms, which had been as far as possible extracted with ether, were repeatedly boiled under a reflux with absolute alcohol. The hot solution was filtered from insoluble particles, and from the turbid filtrate on cooling an amorphous powder immediately precipitated on the walls and bottom of the containing vessel. The clear liquid was decanted.

The solid powder was recrystallized from absolute alcohol; on long standing hard shiny crystals were deposited from the solution. Glacial acetic acid and methyl alcohol were found better solvents for crystallization. The melting-point of the crystals was taken. At 97° they began to shrink, at 110° ran down the tube, at $120\text{--}142^\circ$ water was given off and at 150° the substance seemed to solidify again and at 204° it finally melted. This substance from its properties was obviously trehalose, the occurrence of which had been observed many years ago in this mushroom by Winterstein [1894].

The acetate which is best crystallized from boiling absolute alcohol melted at 102° . $[\alpha]_D^{16} = +159.83^\circ$ (chloroform).

The octabenzoate was made by shaking an aqueous solution of the trehalose with benzoyl chloride and 10% NaOH. A sticky oily solid separated out, which was filtered off, and after washing with water dissolved in a mixture of alcohol

and ether from which, on concentration, crystals were obtained. The crystals were recrystallized several times from boiling alcohol, but as this did not seem to produce a pure substance, the alcoholic solution was boiled up for 30 min. with animal charcoal, filtered, concentrated and left to stand. The crystals which were deposited from the solution had a melting-point of 177°.

SUMMARY

The sterols of *Boletus edulis* consist mainly of ergosterol together with small quantities of a sterol which closely resembles spinasterol.

The main crystallizable carbohydrate isolated was trehalose.

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