CLIII. A POLYSACCHARIDE FROM THE POLLEN OF TIMOTHY GRASS (*PHLEUM PRATENSE*).

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RECENT investigations of the relationship of various constituents of pollens to the allergic diseases for which they are responsible have indicated that, in most cases, the active principles are associated with the protein fractions. In an examination of the fractions of Timothy grass pollen an attempt was made to isolate a polysaccharide on account of the possibility that such a compound might play a part similar to those of the specific soluble carbohydrates isolated from various pathogenic bacteria.

By use of the methods employed in the isolation of the specific carbohydrate from the tubercle bacillus it has been possible to isolate from the pollen a laevorotatory complex carbohydrate which appears to be of the same chemical type as the corresponding bacterial substances. A preliminary chemical examination has shown that it yields *l*-arabinose, galactose, and a non-reducing acid on hydrolysis with acids, thus displaying a certain similarity to the specific carbohydrate of the tubercle bacillus. The relationship of the polysaccharide to the allergic reactions of the protein fractions of the pollen is being examined.

It is improbable that the polysaccharide is in any way connected with the glucoside dactylin, isolated by Moore and Moore [1931] by direct aqueous extraction of Timothy and Orchard grass pollens, since dactylin gives no pentose reactions. There is very little recorded concerning the occurrence of gum-like carbohydrates in other pollens; Johnson and Rappaport [1932] state that no such substance could be isolated from ragweed pollen, whilst Miyake [1922] describes the isolation of 5.7 % of a pentosan from maize pollen. Black [1931] isolated from the pollen of the giant ragweed a carbohydrate containing 5.2 % N which reacted specifically in the skin and nasal mucosa of ragweed-sensitive persons.

EXPERIMENTAL.

Preparation of the carbohydrate.—10 g. of pollen (*Phleum pratense* collected in 1930) were digested with 4 % sodium hydroxide (200 cc.) on the water-bath for 6 hours. At the end of this period some of the cells were broken, but most were only swollen and slightly ruptured. The liquid was diluted with an equal

volume of water, acidified with acetic acid and filtered. The filtrate was treated with an excess of lead acetate and the precipitate, which contained most of the yellow colouring matter but gave no Molisch reaction, was removed and discarded. Basic lead acetate and sufficient ammonia to basify the liquid strongly were then added and the heavy precipitate, which contained all the substance responsible for the Molisch reaction, was removed, washed with water and suspended in dilute acetic acid. After stirring mechanically for several hours the insoluble material was removed and discarded and the filtrate treated with hydrogen sulphide. The lead-free liquid was then evaporated at 40° to a small volume and poured into ten volumes of ethyl alcohol. The precipitated solid was dissolved in water (8 cc.), treated with an equal volume of methyl alcohol and the precipitated material filtered off. When thus isolated, the material insoluble in 50 % methyl alcohol was only slightly soluble in cold and somewhat more so in hot water. It gave no coloration with iodine and did not reduce Fehling's solution. Upon hydrolysis with acids reducing sugars were formed.

The water-soluble carbohydrate was obtained by pouring the filtrate from the above material into an excess of methyl alcohol (80 cc.) and was further purified by another precipitation from 50 % methyl alcohol.

Two other preparations were made, one from pollen (30 g.) which had been ground in a ball-mill and extracted with dilute saline and the other from pollen (55 g.) which had previously been extracted with acetone. The yields were 1.1 and 1.9 g., respectively. In the last preparation the treatment with sodium hydroxide was prolonged until most of the pollen grains had burst (32 hours).

The preparations formed white powders which were very soluble in water and which changed to gummy masses on addition of a few drops of water. The last two preparations had respectively $[\alpha]_{5461} - 42.0^\circ$ and $- 39.1^\circ$ in 1 % aqueous solution. A fractional precipitation experiment was made to test the homogeneity of the material; a solution of the third preparation (0.2 g.) in water (1.5 cc.) was treated with alcohol (4.5 cc.), the precipitated material (0.06 g.) removed, the filtrate poured into an excess of alcohol (60 cc.), and the second fraction collected (0.13 g.). The first fraction had $[\alpha]_{5461} - 34.2^\circ$ and the second $- 39.1^\circ$. The fractions contained 0.9 and 0.5 % N. The unfractionated preparation contained 0.2 % P.

The carbohydrate gave strong characteristic reactions for pentoses with hydrochloric acid and phloroglucinol and with hydrochloric acid and orcinol. No reaction for methylpentoses could be obtained by the reaction of Rosenthaler [1909]. No reaction for glycuronic acid was obtained using the modified method of Neuberg and Kobel [1931]. The carbohydrate possessed only a very faint reducing power towards Fehling's solution, but after warming for a few minutes with dilute hydrochloric acid much reducing sugar was formed.

An experiment was made on the rate of hydrolysis of the carbohydrate in sulphuric acid in order to determine whether the sugars were liberated from the complex in stages or not. 7.908 mg. of the carbohydrate were dissolved in 1 % sulphuric acid (200 cc.) and 10 cc. portions of this solution were heated in

a boiling water-bath for periods up to $4\frac{1}{2}$ hours. At the end of the chosen period each tube was cooled quickly and immediately neutralised with the appropriate amount of *N* sodium hydroxide. The amounts of reducing sugars formed were determined as glucose by the Hagedorn-Jensen method.

Tube	Time mins.	% Reducing sugar formed
1	0	4.5
2	5	12.4
3	10	32.6
4	12	34.9
5	20	36.2
6	40	42.8
7	60	45.3
8	80	45.8
9	120	46.5
10	160	48.3
11	220	52.4
12	260	50.1

The values obtained show that reducing sugars are rapidly formed during the first hour of hydrolysis and that after that time a further slow formation of reducing substances occurs which ceases after about four hours.

For the purpose of examination of the products of hydrolysis, 1.1779 g. of the carbohydrate were heated on the water-bath for 2 hours in 100 cc. of 1 % sulphuric acid. The observed rotation ($\lambda = 5461$, $l = 1$ dm.) changed during this time from -0.46° to $+0.64^\circ$. The liquid was then digested with an excess of barium carbonate until neutral to litmus paper. After filtration the liquid, which contained barium ions, was evaporated to a small volume and poured into ten volumes of methyl alcohol. The precipitated material (0.18 g.; Ba, 27.0 % on air-dried material) did not reduce Fehling's solution and gave no naphthoresorcinol reaction. The methyl alcohol-soluble portion was evaporated to a small volume and again treated with methyl alcohol; a trace of insoluble material was removed. The methyl alcohol was finally removed and the residue evaporated several times with water. The residual syrup gave the deep red colour characteristic of pentoses upon distillation with 12 % hydrochloric acid and pouring the distillate into a solution of aniline acetate. Attempts were made to form hydrazones with the following hydrazines: phenylhydrazine, *as*-phenylmethylhydrazine, *as*-benzylphenylhydrazine, *p*-bromophenylhydrazine, and *as*-diphenylhydrazine. Phenylhydrazine gave a small amount of a substance freely soluble in water and crystallising in large needles; it appeared to be quite distinct from mannose phenylhydrazone; *p*-bromophenylhydrazine and phenylmethylhydrazine gave clusters of fine needles similar in appearance to the galactose compounds. Diphenylhydrazine and benzylphenylhydrazine gave comparatively large amounts of crystalline products. More of the diphenylhydrazone was prepared and washed with alcohol and water. It had m.p. $197-199^\circ$ (decomp.) whilst a sample of authentic *L*-arabinose diphenylhydrazone melted at $199-202^\circ$ (decomp.); Muther and Tollens [1904] give m.p. $204-205^\circ$ (decomp.). A solution of this diphenylhydrazone (0.0216 g.) in

pyridine (2 cc.) and alcohol (3 cc.) had $\alpha_{5461} + 0.36^\circ$ ($l = 2$ dm.), whilst the authentic specimen (0.0212 g.) had $+ 0.34^\circ$ under the same conditions.

Since the appearance of the phenylmethylhydrazone suggested the presence of galactose a portion of the sugar was oxidised with 25 % nitric acid and a small amount of mucic acid was obtained (m.p. $210-212^\circ$ decomp. when the tube was inserted at 200° and the bath heated fairly rapidly). Oxidation of the original carbohydrate (0.1 g.) also yielded mucic acid (0.0104 g.)

The barium salt (0.1 g.) was subjected to further treatment with 2*N* sulphuric acid (10 cc.) for 16 hours. A small amount of reducing sugar (0.05 g.) was formed and some barium salt recovered (0.03 g.) which gave no naphthoresorcinol reaction. The sugar fraction was divided into two portions one being treated with diphenylhydrazine and the other oxidised with nitric acid. A small amount of mucic acid was obtained, but no arabinose diphenylhydrazone could be isolated.

The recovered barium salt lost 8.17 % of its weight on drying at 110° and contained 30.2 % of barium (calculated on the dried material).

Upon concentration of the alcoholic filtrate from the first precipitation of the carbohydrate a small amount of crystalline material separated in a form which suggested that it might be inositol. After recrystallisation of this substance (0.02 g.) from a small amount of aqueous alcohol it had m.p. 223° alone or mixed with an authentic specimen of inositol.

SUMMARY.

The preparation of a gum-like polysaccharide from the pollen of Timothy grass is described. Upon hydrolysis with acids *l*-arabinose, galactose and a non-reducing acid are produced.

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