CLXXXI. ON THE ALLEGED AUTOHYDROLYSIS OF VEGETABLE IVORY.

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VEGETABLE IVORY (*Phytelephas macrocarpa*), the endosperm of the seed of the tagua palm, has long been known as the most convenient source for the preparation of mannose. This sugar occurs in nature in the form of mannans (polysaccharide condensation products) which on acid hydrolysis yield the simple hexose. Approximately 75 % of the vegetable ivory is carbohydrate.

In view of these facts Paton *et al.* [1924] postulated the presence of an enzyme in the ivory nut shavings capable of hydrolysing the insoluble mannans present. The shavings were suspended in water (1-20) and incubated at 45° (optimum temperature) with the addition of toluene to prevent bacterial contamination. A mannose trisaccharide was believed to be first liberated and finally after 10 days only mannose was produced. In neither case was the mono- or tri-saccharide isolated as such; their determinations were solely through formation of corresponding phenylhydrazone and osazone derivatives. Their experiments for enzymic activity may be severely criticised in that no control studies were attempted.

The original purpose of the study reported in the present paper was to investigate the nature of the enzyme and to obtain the liberated mannose, as such, if present at all. However, in the course of the investigation, conclusive evidence accumulated indicating that no mannanase exists in vegetable ivory; and that the presence of a reducing substance in the filtrate is due simply to a warm (45°) aqueous extraction of the nut meal. The carbohydrate responsible for the reducing action of the filtrate was isolated and its properties determined. It is not mannose but apparently a simple derivative of this monosaccharide, which on acid hydrolysis is converted quantitatively into mannose.

EXPERIMENTAL PART.

Attempts to demonstrate the presence of a mannanase.

In order to demonstrate whether the attempts to purify an enzyme or to augment its activity have been successful, it is essential to have a suitable substrate which can be subjected to the action of the preparations. Possibly a mannan might have been used in our work. However, we preferred to use whole vegetable ivory treated in such a manner as to destroy any enzyme that may be present. Boiling an aqueous suspension of the ivory nut meal for 1 hour, followed by incubation of the filtered material at 105° for 10 days was found to be adequate, in that an additional 10-day incubation of the washed material at 45° in accordance with the method of Paton *et al.* [1924] failed to yield any reducing substance in the filtrate. This material, called "treated" vegetable ivory in this report, was washed free of reducing substance and dried in a vacuum desiccator at room temperature.

Tests for enzymic activity (10-day incubation of the aqueous extract at 45°) were conducted upon washed vegetable ivory meal in the presence of equal concentrations (0.42 %) of toluene, thymol and mercuric chloride. The yield of reducing substance, calculated as mannose, was determined by use of Benedict's [1911] copper titration method. Simultaneously with these, two other experiments, wherein additional equal amounts of treated vegetable ivory were added to the incubation flasks, were carried out. Assuming an enzyme to be present, and 10 days to be adequate for completion of the enzymic hydrolysis [Paton *et al.*, 1924], the addition of an equivalent amount of the substrate to the action of the enzyme in the vegetable ivory should result in the liberation of more reducing substance as compared with the controls. The fact that there was an equal liberation of this compound at the conclusion of the incubations (0.88 % yield, calculated as mannose) argues against the presence of a mannanase in vegetable ivory.

Paton *et al.* [1924] determined the reducing powers of different solutions after 2 hours' incubation by titration with Fehling's solution and constructed a curve from which the optimum p_H for the enzyme action was read. However, they failed to take into account that merely washing vegetable ivory with water at room temperature yields a filtrate possessing considerable reducing power. Therefore, might it not be possible that the optimum p_H , as determined by these workers, is merely that for the aqueous extraction of vegetable ivory? In our experiments we had begun with vegetable ivory washed until the runnings were free of reducing substance, so that any additional reducing material obtained upon the completion of the incubation might be attributed to the action of the enzyme.

Might it be possible that during the washings we had also removed the enzyme? If such had been the case, the incubation of "whole" (original unwashed non-heated) vegetable ivory should yield a filtrate containing a greater amount of reducing substance than that liberated in the washed meal *plus* that present in the washings of the meal. Such results were not obtained. An equivalent yield of reducing substance (1.30 %, calculated as mannose) was obtained in both cases. These findings indicate that no mannanase had been removed in the preliminary washing of the ivory nut meal.

Furthermore, the original investigators state that "at a temperature of 60° the action (of the enzyme) appeared to be almost completely annulled". However, we found that the incubation of an aqueous suspension of the nut meal at 65–70° yielded considerable amounts of reducing substance and the addition of washed vegetable ivory to boiling water resulted within 10 minutes in the liberation of this compound; and this occurred at a temperature at which enzymes do not function.

Investigations as to the chemical nature of enzymes have as yet been very indecisive. On the whole, two viewpoints exist. The American school represented by Sumner, Northrop and Sherman, who have prepared crystalline enzymes, advance the conception that enzymes are protein in character. The view of Willstätter's school is that enzymes are composed of a specific active group and a colloidal bearer or carrier and that the specific group binds the enzyme to the substrate.

With these views in mind, we attempted both through standard protein preparations and adsorption technique to secure active fractions or concentrates of the enzyme alleged to exist by Paton and co-workers. A 5 % saline extract of the fat-free meal was saturated with $(NH_4)_2SO_4$ to precipitate the globulin and albumin fractions. The precipitated proteins were subjected to dialysis in running water for 10 days until a negative sulphate test was obtained, toluene being

used as a preservative. The precipitated globulins were centrifuged off, dehydrated and dried. The supernatant fluid was evaporated to a small volume at 45° and the albumin fraction precipitated by 95 % ethyl alcohol. Whole vegetable ivory has 0.697 % N, equivalent to 4.35 % protein. The globulin fraction (14.2 % N) approximated to a 20 % yield and the albumin fraction (11.7 % N) to a 7 % yield, calculated as percentage of total protein. The protein fractions gave low Kjeldahl nitrogen values, but these determinations were carried out upon the crude products and were not corrected for contaminants.

After a 1-hour extraction at 45° of an aqueous suspension of ivory nut meal buffered at p_H 4.2, 50 g. activated carbon "nuchar"¹ were added and the mixture allowed to stand at room temperature for 2 days. The adsorbate was then decomposed in aqueous medium at p_H 9.2, and the filtrate evaporated to a small volume. The final filtrate apparently contained the proteins described above, giving the characteristic protein colour reactions. Due care was exercised during the preparation of the proteins and adsorbate not to permit the temperature of manipulations to exceed 45°.

The adsorption concentrate and the proteins isolated were tested for enzymic activity, using the treated vegetable ivory as a substrate. No reducing substance was liberated under these conditions.

Isolation and properties of the reducing substance.

Two kg. of the washed ivory nut meal were suspended in 20 l. distilled water and incubated as described above. After 10 days the filtrate was boiled, filtered and concentrated *in vacuo* to 50 ml., keeping the temperature of the water-bath below 80°. Throughout the concentration, norite treatments and filtrations were necessary to obtain a clear filtrate. The reducing substance was precipitated from solution by daily additions of 20 ml. glacial acetic acid. The final acetic acid filtrate was practically free from reducing substance, indicating that most of the carbohydrate present in the syrup had been brought down. The precipitate was filtered, washed free of acid with 95 % alcohol, dehydrated with acetone and dried in a vacuum desiccator. 19.0 g. were obtained, indicating a yield of 0.95 %.

Properties of the carbohydrate.

Physical:

- (a) Amorphous and hygroscopic.
- (b) Decomposes at 145–155°.
- (c) Mutarotates; at equilibrium $[\alpha]_D^{22} - 9.62^\circ$.
- (d) Molecular weight (Beckmann), 183.

Chemical:

- (a) Forms glucosazone, m.p. 198°.
- (b) Reducing activity equal to approximately one-third that of mannose.
- (c) On hydrolysis is converted quantitatively into mannose as evidenced by: (1) $[\alpha]_D^{22}$ of the hydrolysate + 14.75°; (2) recovery of 92.7 % of the material in the form of the insoluble hydrazone (m.p. 188°) and osazone (glucosazone) of mannose (m.p. 198°).
- (d) Elementary composition (sodium fusion tests) indicated no nitrogen or phosphorus to be present, a slight trace of sulphur, which, as sulphate, was probably present as an impurity.

In view of the fact that this carbohydrate possesses approximately one-third the reducing ability of mannose, all yields of reducing substance calculated as mannose, reported above, are actually three times as large. This reducing activity

¹ From the Industrial Chemical Sales Co., New York, N.Y., U.S.A.

may be intrinsic or due to the aqueous hydrolysis of the compound, in that boiling the substance with phenylhydrazine hydrochloride and sodium acetate in appropriate proportions produces the crystalline osazone of mannose (glucosazone). The optical activity of the solutions and the identities of the derivatives (through melting-points and mixed melting-points) were carefully controlled, using crystalline mannose prepared by the method of Hudson and Sawyer [1917] as subsequently improved by Horton [1921], Clark [1922] and Levene [1935].

The water-soluble reducing substance in the cold water extract of the nut meal (preliminary washings) was also identified as being the compound described above.

This carbohydrate has, as yet, not been identified with any reported in the literature.

SUMMARY.

1. The presence of a mannanase in vegetable ivory could not be confirmed.
2. During the course of the investigation two new proteins were isolated, namely, albumin and globulin fractions. Further investigation concerning the chemical nature and biological value of these proteins will be undertaken in our laboratory.
3. The reducing substance in the incubation mixtures was isolated and found to be a simple derivative of mannose (probably a simple mannoside or lactone) which on acid hydrolysis is converted quantitatively into mannose.
4. The presence of the reducing substance in the incubation mixture was due merely to its ready solubility in the warm (45°) aqueous extract of the vegetable ivory. The observation that the "treated" vegetable ivory yielded no reducing substance is explained as due to the prolonged heating, followed by washings, resulting in removal of all the water-soluble reducing substance from the outer layer of the particles. This was confirmed by positive reducing tests obtained on the treated nut meal after being reground and incubated at 45° for 10 days.

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