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INULIN IN THE GREEN ALGA *BATOPHORA OERSTEDI* J. AG. (DASYCLADALES)

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C. NÄGELI, in 1863, was the first to notice spherocrystals of the inulin type, practically indistinguishable from those of *Dahlia variabilis*, in alcohol-treated plants of *Acetabularia mediterranea* Lam., a member of the *Dasycladales*. The claim that these crystals are indeed composed of inulin was made by LEITGEB, in 1887, but on insufficient grounds. It was not until 1953 that DU MÉRAC succeeded in isolating 3.5 g of a clearly identified inulin as a white powder from 150 g of fresh *A. mediterranea*; the main criteria she applied for establishing the chemical identity of the product were its optical rotation and specific gravity combined with its ability to yield only fructose upon hydrolysis. In *Dasycladus vermicularis* (Scopoli) Krasser, DU MÉRAC (1955) found no inulin, although she was able to demonstrate at least 5 low-molecular-weight fructosans in addition to free fructose and traces of sucrose. In *Cymopolia barbata* L. Harv. she found sucrose, fructose, and at least 8 low-molecular-weight fructosans (DU MÉRAC, 1956).

These results suggest that the *Dasycladales* form a natural order not only in a morphological-taxonomic sense, but also biochemically: they appear to be characterized by the formation of fructose-polymers as reserve-materials. However, in order to be safe, generalizations should be based on at least a fair number of representative cases. The present author has studied *Batophora oerstedi* J. Ag. for that reason. From this alga also, he could isolate a substance which, according to the criteria applied by DU MERAC, could only be inulin. In addition, however, the compound was "fingerprinted" by means of its X-ray diffraction diagram, and some of its fermentability characteristics were established. Free fructose and fructose-containing oligosaccharides are present in addition to the inulin.

The present investigation raises to 4 the number of cases studied in the *Dasycladales*. Applying the label "fructosan-algae" to the group as a whole may now be somewhat more justified than it was before.

EXPERIMENTAL

A. *Material*

On June 12, 1962, around 5 p.m. (presumably after a long period of photosynthesis), *Batophora* plants were collected in the Lake St. Francis potholes, Bitter Lake National Wildlife Refuge, U.S.A., by Dr. Vernon Proctor, who generously put the material at the present

author's disposal in dried form. The water in the potholes is brackish and the rocky substrate to whicha the lgae are attached is rich in marl. No complete separation could be achieved between the algal material and particles of the substrate, a circumstance which (together with the presence of salt) may be responsible for the high ash content found (around 50 % in some samples; largely CaO). According to FRITSCH (1935), *Batophora oerstedi* plants are not themselves impregnated with calcium compounds.

B. *Isolation and characterization of inulin*

1. *Isolation.* After exhaustive extraction with ethanol (see below), 30 grams of dry, powdered *Batophora* material is treated 3 times with a large excess of water at 90–95° C, under constant stirring; duration of first water-extraction 2 hours, of second and third extraction each one hour. The supernatants (about 1.5 l) are collected by centrifugation, combined, and treated with a 10 % solution of lead subacetate until no further precipitate appears. Centrifugation yields a water-clear, practically colorless fluid, which is treated with a large excess of hydrogen sulfide; the resulting lead sulfide is filtered off, and residual H_2S is eliminated by bubbling air through. The pH now is 4.6. After cooling in ice-water, a large quantity of the cation exchanger IR 120 is introduced into the fluid, where it is left for 30 minutes with constant stirring. After eliminating the cation exchanger, a 3.5-fold volume of ethanol is added to the fluid. The resulting snow-white precipitate appears under the microscope to consist of small spheres or droplets, highly refractive and remarkably uniform in size (2–3 μ); the individual droplets may coalesce in small groups but never form large flakes. After one night in the refrigerator, the precipitate (which has now settled) is washed several times with ethanol. It is dissolved (with ease) in hot water, yielding an opalescent and tasteless fluid. The alcohol precipitation is repeated twice. The compound is thereafter dissolved in a small volume of warm water; the fluid is cooled and allowed to stand at 0° C for an extended period, a procedure resulting in gradual reprecipitation of the substance. Finally, the latter is collected in the centrifuge and dried thoroughly at 80° C.

As an alternative procedure, deproteinized solutions can be provided with an anion exchanger such as IR 4B or IR 400. As soon as the pH approaches neutrality, an abundant snow-white precipitate forms, composed chiefly of a calcium-fructosan compound. The metal in it is later eliminated as Ca-oxalate; subsequent application of ion exchangers leaves the purified inulin.

The yields of inulin varied for different samples from 6.5 to 12.5 % of the organic matter of the alga, "organic matter" being defined somewhat arbitrarily as dry weight (before alcohol-extraction) minus ash.

2. *Properties of the product.* The isolated compound gave positive Seliwanoff and diphenylamine/HCl reactions (see RADT, 1928). Its specific gravity, determined at room temperature (22° C) in a pycno-

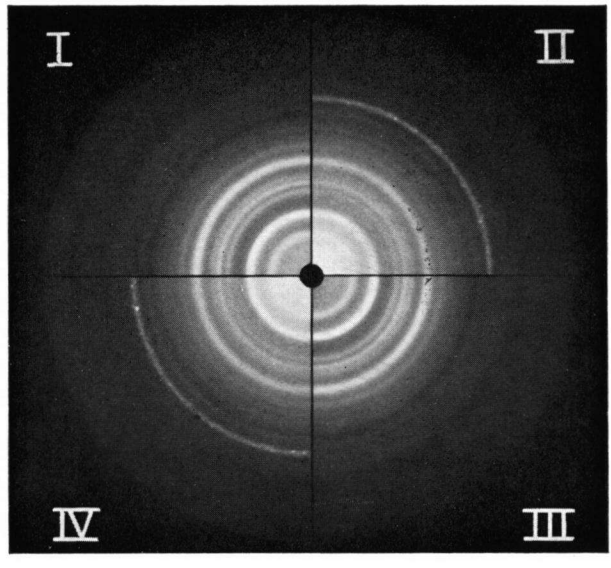

Fig. 1. Quadrants of X-ray powder diagrams of commercial higher plant inulin (I and III) and of *Batophora* fructosan (II and IV). CuK_α radiation: distance specimen to film 40 mm; pinh. 0.5 × 40 mm.

meter with ethanol as the intermediate, was 1.55. It displayed no sharp melting-point, but began to brown and decompose at about 180° C. The substance was insoluble in alcohol, other organic solvents, and cold water, but dissolved readily in hot water. Upon cooling, it precipitated again; the more concentrated the solution, the sooner. $[a]_D$, determined in a 5 cm tube at room temperature, — 40°. Acid hydrolysis (0.1 ml concentrated H_2SO_4 + 10 ml of a 2.5 % solution, 10 minutes in a boiling water bath) produced a reducing compound which, heated with Na-acetate and phenylhydrazine.HCl, yielded the characteristic yellow needle-clusters of phenylglucosazone or fructosazone (melting-point, after several recrystallizations, 205° C). $[a]^{20}_D$ of the hydrolysis product, calculated on the basis of reducing power present, — 89°, indicative of fructose. Extended treatment with dilute mineral acid at 100° C (Sieben-method; compare LEHMANN, 1931) destroyed the hydrolysis product almost quantitatively, again indicative of fructose. Indeed, filter paper chromatography (for details, see below) revealed only this sugar. The polysaccharide was fermented by *Saccharomyces fragilis* Jörgensen but not by *Sacch. cerevisiae* Hansen (Durham tubes; a 2 % solution, 24 hours at 30° C; see SNYDER and PHAFF, 1960). It was split slowly by very high concentrations of invertase at pH 6. Its X-ray diffraction pattern (see figure) was identical with that of a pure inulin from Merck, except for an additional peripheral ring which must be ascribed to a remaining slight impurity. There is good agreement between the pattern and the inulin-patterns described in the literature (FUCHS; KATZ and DERKSEN; KATZ and WEIDINGER).

C. *Free fructose and oligosaccharides*

20 g of thoroughly dried *Batophora* powder is extracted exhaustively by refluxing with 80 % ethanol (4 × 450 ml, 2 hrs. each time). The combined extracts are concentrated under reduced pressure to about 50 ml, and a chlorophyllous precipitate is eliminated in the high speed centrifuge (10 minutes at 12,000 × g). The olive, opalescent supernatant (pH 6.6) is acidified with dilute acetic acid to give a pH of 4.7 and again spun in the high speed head (10 min. at 12,000 × g) to eliminate more dark-green material. The supernatant, olive-grown with greenish hue and still somewhat opalescent, contains the mono- and oligosaccharides. Heated with Na/acetate and phenylhydrazine.HCl, it produces only phenylglucosazone (melting-point, after several recrystallizations, 205° C). A fructose determination according to ROE (1934), applied to the 100-fold diluted extract, reveals 157 γ of ketose per ml, corresponding with 7.1 g of "fructose" per 100 g of organic matter present. Assay for total carbohydrate with the anthrone reagent (which responds equally well to fructose and glucose; see SATTLER and ZERBAN, 1948 and also MORRIS, 1948) yields 149γ. The excellent agreement between the 2 methods seems to indicate that all the sugar-residues present are fructose units. However, the limitations of the anthrone method should be taken into account; the total absence of glucose (e.g., in the form of sucrose) must be

considered unlikely, and traces of this compound may well have escaped notice. It should also be remembered that the inulin-molecule (consisting of about 35 fructose units) terminates in a glucose molecule (WHISTLER and SMART, 1953). Determinations of the reducing power with the Luff-Schoorl reagent gave fructose-values markedly lower than those indicated by the Roe method (e.g., 10.4 mg according to Luff-Schoorl, 15.0 mg according to Roe). The difference was partly eliminated by prolonged treatment with invertase, and almost completely by hydrolysis with dilute mineral acid at 100° C for 10 minutes. This is indicative of the presence of fructose-containing oligosaccharides. More conclusively, the latter were revealed by filter paper chromatography (Whatman No. 1 filter paper; running-fluids butanol/acetic acid/water 4:1:5, upper layer, or isopropanol/acetic acid/water 7:1:2; reference compounds fructose, sucrose, raffinose and stachyose; developer urea-phosphoric acid; see WISE *et al.*, 1955). Present (in addition to much fructose): a trace of sucrose(?) and 3 oligosaccharides with R_F values between 0 and the R_F of raffinose. The conclusion must be, that *Batophora oerstedi* is intermediate between *Acetabularia mediterranea* and *Dasycladus vermiformis*, in that it possesses both inulin and low-molecular-weight fructosans.

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