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Fructose and Fructose-Anhydrides from *Dahlia* Inulin

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ABSTRACT

Inulin from *Dahlia* spp. tubers (50 g% yield, dry basis; 12.5 g% yield, wet basis) competes with Jerusalem artichoke and chicory as a source of fructose. Mild acid hydrolysis under pH control instead of nominal acid concentration of aqueous orthophosphoric acid resulted in almost quantitative monomerization of the labile fructan backbone. Byproducts hydroxymethylfurfural (HMF), (di)fructose anhydrides (DFA), and oligosaccharides were minimized. Microflora were collected from rotting *Dahlia,* and an inuloytic mold, able to metabolize HMF, as well as bacteria-producing DFA III were recovered. Microbiologically synthesized DFA was tested for biological activity using isolated rat liver perfusion.

Index Entries: *Dahlia;* inulin; fructose; difructose anhydride; perfusion.

INTRODUCTION

Sweeteners are finding a permanent and increasingly important place in the world market. Fructose, a naturally occurring monosaccharide, surpasses sucrose in sweetness. Large-scale production of fructose is based on starch hydrolysis followed by isomerization leading to a 1:1 mixture of glucose and fructose. This initial product may then be enriched to high-

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fructose corn syrup (HFCS) by progressive removal of glucose. This is a relatively expensive step. HFCS makes up over 1/3 of the US sweetener market (*1*).

Inulin, which accumulates in the tuberous roots of *Dahlia* and Jerusalem artichoke, may produce, on complete hydrolysis, a mixture of fructose:glucose of about 30:1. This is because of the existence of a single sucrosyl group in one of two nonreducing end groups of the unbranched, short, and extremely labile fructan chain. Inulin hydrolysis may be carried out with several types of microorganisms (molds, yeasts, bacteria) or mild acid conditions. The latter requires small amounts of mineral acid(s) and a temperature of $< 100°C$. The major limitation of the acid hydrolysis is the ease of hydroxymethylfurfural (HMF) generation from the released fructose. Also, the acid catalyst must be neutralized, forming insoluble salts, such as calcium sulfate. Usually the strongest mineral acids (sulfuric and hydrochloric) are used as hydrolytic agents of inulin. One report (*2*) and one expired patent (*3*) focused on the use of HCl, but included also the mention of phosphoric acid. Ortophosphoric acid (OPA) has been employed by us at 0.1–0.2% under moderate thermopressurization for the complete monomerization of sugarcane hemicelluloses (*4*). However, the β-(1,4)-xylobiosyl structural unit of the hemicellulose is less acid-labile than the β-(2,1)-inulobiosyl unit present in inulin. An advantage of aqueous phosphoric acid (pH range 2–4) in comparison to strong mineral acids is the fact that it exempts the removal step and/or neutralization. The industrial processing of Jerusalem artichoke has been the subject of a recent review (*5*). The first focus of our work is the optimization of inulin hydrolysis using phosphoric acid. In addition, we search for a safe fructose syrup, free of HMF and with no restriction arising from other byproducts, such as difructose anhydrides. In comparison to other natural sweeteners (glucose and sucrose), fructose is more soluble, sweeter, and less glucogenic/insulinogenic, but more lipogenic. Despite the above advantages and its direct entry in metabolic routes through two kinases, criticism has been raised against dietary adaptation of this ketose, in view of some changes it provokes (*6*).

Inulin may also be subject to a unique catabolism as detected in a bacterium from the genus *Arthrobacter*, isolated by Japanese researchers (*7*). The polysaccharide is converted to a modified form of the inulobiose unit, the difructose anhydride (DFA III), which corresponds to the 2′,1:2,3′-linked difuranose isomer, owing to the enzymatic loss of 1 mol of water. The DFA III-producing species was identified as *ureafaciens*, and its enzymic apparatus for this inulin transformation was named inulase II (*8*). The enzyme is extracellular and inactive on β-2,6-linked fructans (*9*). DFA III was reported as having sweetening properties (*10*). Inulin acid hydrolysis (sulfuric acid) has resulted in the formation of three different DFAs (*8*), including the type III (*11*).

We included in this work the search for DFA III-producing microorganisms from a likely source, rotting *Dahlia* tubers. We also studied the initial steps of the evaluation of the biological activity of this unusual class of disaccharides.

MATERIALS AND METHODS

Polysaccharide Source and Processing

Dahlia pinnata was selected as the source for inulin. The preparation of the reserve polysaccharide was carried out from cortex-free tubers in order to avoid too deep browning of the inulin preparation. Extractant pH 7.0 phosphate buffer was brought to boiling before receiving the addition of freshly sliced tubers. The hot extract was filtered through a thick layer of cheesecloth to ensure no passage of particulate matter. Inulin overnight insolubilization was allowed in a cold room (8–10°C). Only the well-settled material was washed with ethanol and finally dried with acetone. When needed (e.g., for mol-wt determination), the light, creamy preparation was further clarified with activated charcoal to render a whitish powder. Inulin hydrolysis with orthophosphoric acid (OPA) was carried out in a stainless-steel thermopressurized reactor usually at 135°C and 2.1 atm pressure. OPA was added such that a pH range from 1.5 to 4.0 was maintained. Polysaccharide concentration varied from 10 to 50%, the greater concentration being attained by vigorous mixing in a blendor to a homogeneous paste.

Microorganism Screening and Cultures

Rotting *dahlia* tubers, whose firm inulin-containing pulp turned to a gray-black soft mass, were streaked on a medium containing 1% inulin and 0.1% yeast extract, buffered with 20 m*M* pH 7.0 phosphate, and solidified with 2.0% agar. Fast-growing colonies were selected for further growth in liquid medium to be used for the production of hydrolytic products other than oligosaccharides. Those microorganisms releasing fructose exclusively were selected as well those producing compounds with selective chromatographic properties. The latter criteria were: (a) movement ahead D-fructose when using silica gel plates and ethyl acetate-based solvent in the thin-layer chromatography (TLC) run; (b) orange to light brown color when reacting with the orcinol/sulfuric spray. Liquid culture of the DFA III-producing reference strain (*Arthrobacter ureafaciens* ATCC 7562) and isolates with the same biochemical ability were scaled up to 5 L in order to produce gram amounts of extracellular dianhydride. For this stage, the salt medium of Nakamura et al. (*12*) was reinforced with 0.05% yeast extract.

The crude product was sequentially purified by sorption/desorption of the 10× concentrated medium on activated charcoal (fast elution with 20% ethanol) and by batch chromatography of its syrup on a silica gel column with acetone and acetone-methanol mixtures (1:1 to 1:2) as eluant.

Analytical Procedures

Fast profile of fructose and fructose-derived compound solutions was obtained by TLC in silica gel G 60 with selected solvents to ensure improved resolution:

A. Isopropanol:ethyl acetate:water (85:5:10);
B. Isopropanol:nitromethane: ethyl acetate:methylethylketone:water (50:45:50:25:30); and
C. Chloroform:methanol:methylethylketone (8:1:1).

The latter solvent system was used for the complete separation between HMF and its immediate product of biodegradation by an *Aspergillus* isolate. Chromatographic plates, after UV light examination for HMF, were developed with 0.5% orcinol in methanol:sulfuric acid (95:5) with constant inspection during the 3–10 min heating at 100°C. A DFA III standard (100 mg) was kindly provided by Kuniji Tanaka (Osaka Kyoiku University, Japan).

Ketosaccharide composition was also assessed as persylyl derivatives by GLC or GLC-MS (Gas liquid chromatography-mass spectrometry) using a SE-30 capillary methylsilicone column or a capillary PONA dimethylsilicone column, respectively. High-performance liquid chromatography (HPLC) was carried out with these conditions: a Waters/Millipore SC 600E/Wisp 712 with a 410 DR assembly, and a CAC column irrigated with acetonitrile:methanol (8:2) at 3.5 mL/min flow (612 psi). Inulin mol-wt determination was performed by gel permeation chromatography (GPC) at CERMAV, Grenoble, France, using Shodex columns.

Mutagenic Test and Isolated Rat Liver Perfusion

DFA III was submitted to the reverse mutation assay using different auxotrophic mutant strains of *Salmonella typhimurium* as described by Maron and Ames (*13*). Male albino rats (Wistar strain, 200–250 g) were starved for 24 h before the surgical removal of the liver under pentobarbital anesthesia. The perfusion technique described by Scholz et al. (*14*) was used in an apparatus built at Maringa State University. The perfusion fluid was pH 7.4 Krebs/Henseleit bicarbonate buffer, saturated with an oxygen:carbon dioxide mixture (95:5%) and containing 25 mg% bovine serum albumin. Fructose and DFA III were directly dissolved in the perfusion fluid. The fluid was pumped through a temperature-regulated (37°C) membrane oxygenator prior to entering the liver via a canula inserted in the portal vein. Oxygen concentration in the venous perfusate

was monitored continuously, employing a teflon-shielded Pt electrode, and its uptake rate was calculated from the arterio-venous concentration differences and the flow rate. The rates of metabolite release (glucose, lactate, pyruvate) were calculated from the venous concentrations and the flow rate. All rates were referred to the wet wt of the liver. Samples of the effluent perfusion fluid were collected in 2-min intervals and analyzed for D-glucose, L-lactate, and pyruvate by standard enzymatic procedures (*15*). DFA III presence in the perfusate was assessed by the TLC procedure.

RESULTS AND DISCUSSION

Our inulin preparation may represent a true native polymer, since mol-wt determination by GPC indicated a $M_w = 4300$ (ca. 27 anhydrofructose residues). The tuber, its flour, and crude or clarified inulin did not differ in terms of fructose production, provided the mild hydrolysis with aqueous phosphoric acid was carried out under pH control at the time of reactant mix instead of the use of nominal acid concentration. Whole (cortex-free) tuber or its flour (water suspension showing a pH close to 7.0) took up more OPA than expected for a particular desired pH, probably because of the protein and hemicellulose native contents. Differences among the hydrolysates of tuber and purified inulin were mainly owing to the degree of pigmentation of the hydrolysates. A typical result of such hydrolysis tests is shown in the TLC analysis of Fig. 1. Thermopressurization reached 135°C and 2 atm for a 2-min residence time for individual assays previously adjusted to the pH range from 1 to 3.4. Tuber (left) and flour (right) were compared. Taking the solvolytic or the autohydrolytic assay as reference (lane S), most of the pH-controlled assays led to significant coproduction of HMF (the fastest moving spot, $R_f' = 0.58$). HMF was also easily distinguished from fructose or fructose-containing compounds because of its prompt reactivity under heating with the orcinol reagent (yellow→orange→wine→gray-bluish). Fructose ($R_f' = 0.37$) was the main product released under all conditions, but in part of them (e.g., lanes 1*, 4, 4′, and 5′), two additional coproducts were also detected ($R_f'[s] = 0.53$ and 0.05). The faster of these two spots cochromatographed with the DFA III standard, and the identity between them was established by capillary GLC. It is worthy of mention that, in the more productive assay regarding fructose production (Fig. 1, lane 1*; tuber pulp adjusted to pH 3.4), these two substances although in very reduced amounts were also present. HMF was absent as well as oligosaccharides. The conditions of this test were selected as optimal for inulin hydrolysis.

From the screening of inulolytic or inulin-transforming microorganisms interesting biochemical properties resulted as shown in Table 1. Except for the isolate "m" (a mould, probably an *Aspergillus*), all others are gram (−) and (+) bacteria. "P⁻" presented a flat and spread growth with

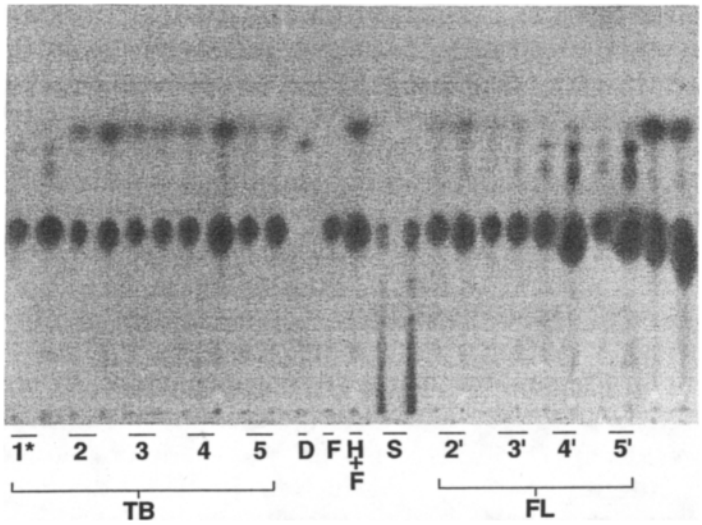

Fig. 1. Thin-layer chromatogram (TLC) of *Dahlia* tuber (TB) or tuber flour (FL) pH-controlled hydrolyses with thermopressurized aqueous phosphoric acid (135°C/2 atm). 1*=hydrolysis at the mildest condition (pH 3.4). 2,3,4,5 and 2′,3′,4′,5′=hydrolysis trials where the pH ranged from 1.5 to 1.0. S=inulin solvolysis (water; no pH control). TB=*Dahlia* tuber (intact; cortex-free). FL=crude inulin flour. Standards: D=difructose anhydride (DFA III). F=frutose. H+F=hydroxymethylfurfural+fructose.

whitish colonies. The remaining isolates, forming gummy colonies, could not be differentiated within the first 48 h of growth. "C^-," and with less efficiency "F^-," appeared to be good producers of a viscous exopolysaccharide at the expense of inulin as C source (the still uncharacterized glycan is built on more than one aldose sugar). "Yel," in the same way as the reference "Au" (*A. ureafaciens*, ATCC 7562), acquired a yellow pigmentation on culture aging (liquid culture showed that both formed a pink-wine pigment with pH-dependent color pattern). Finally, "cr" (from its permanent cream color) was distinct from the pigmented isolates. The latter three bacteria proved to be good DFA III producers. The TLC plate of Fig. 2 (right half) shows anhydride evolution from 1 g% inulin supplemented with 0.1 g% yeast extract after 12 and 22 h at 28°C and 100 rpm; a heavy inocululm, that is, a generous loopful in 20 mL medium, was used. Lesser amounts of product were detectable using Nakamura's medium (*12*), except for the reference strain. A striking feature of the reference strain of *A. ureafaciens* (*16*), also displayed by our bacterial isolates, was its ability to hydrolyze DFA III to fructose in the late phase of growth. The intracellular enzyme responsible for this does not correspond to inulinase or invertase. Confirmation of the DFA chemical structure determined by TLC analysis was obtained by capillary GLC on a SE-30 column at 190°C with a 2-min hold, followed by programming at 20°C/min until 250°C was reached, the following retention times resulting: HMF (3.9 min),

Table 1
Properties of Isolates from Rotting *Dahlia* Tubers[1]

Isolate	Colony aspect	Pigment	Fructose DFA III[2] production from		EPS[3] inulin	HMF[4] catabolism
A.u. 7562	Gummy	+	(+)	+++(+)	+	nd
"yel"	Gummy	+	(+)	++	+	nd
"cr"	Gummy	−	(+)	+++	++	nd
"F"	Gummy	−	(+)	(+)	++	nd
"C"	Gummy	−	++	−	+++	nd
"P"	Flat	−	++	−	(+)	nd
"m"	Mycelial	+[5]	++	−	−	+

[1]Cultures in yeast extract-supplemented inulin; 28°C; 150 rpm.
[2]DFA III = 2,1':2',3-linked difructofuranose anhydride.
[3]EPS = exopolysaccharide.
[4]HMF = hydroxymethylfurfural.
[5] = dark spores.

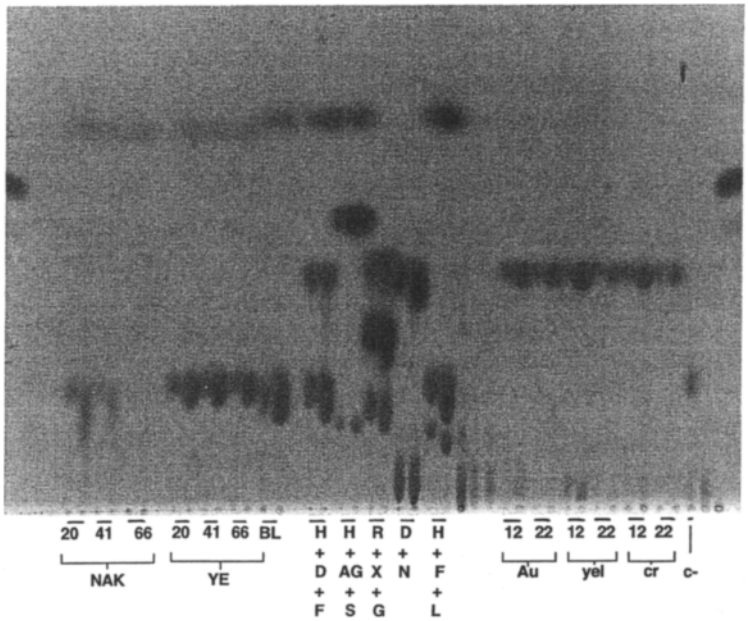

Fig. 2. Thin-layer chromatographic (TLC) analysis of hydroxymethyl (HMF) and inulin-metabolizing isolates. Left half: 20, 41, 66 = culture time (h) of isolate "m" in Nakamura's (NAK) and yeast extract (YE)-based media. BL = (blank); nonincubated culture media (mix of fructose, HM-furfural, salts, and yeast extract). Right half: 12, 22 = culture time (h) of reference *A. ureafaciens* (Au), and isolates "yel," "cr," and "C⁻" in Nakamura's medium reinforced with 0.05 g% yeast extract. Standards (in order of decreasing mobility): H + D + F = HMF + DFA III + fructose. H + AG + S = HMF + 1,6-andryglucose + sucrose. R + X + G = rhamnose + xylose + glucose. D + N = DFA III + rafinose. H + F + L = HMF + fructose + diheterolevulosan (= β-fructopyranose 2,1': 2',1 α-fructopyranose).

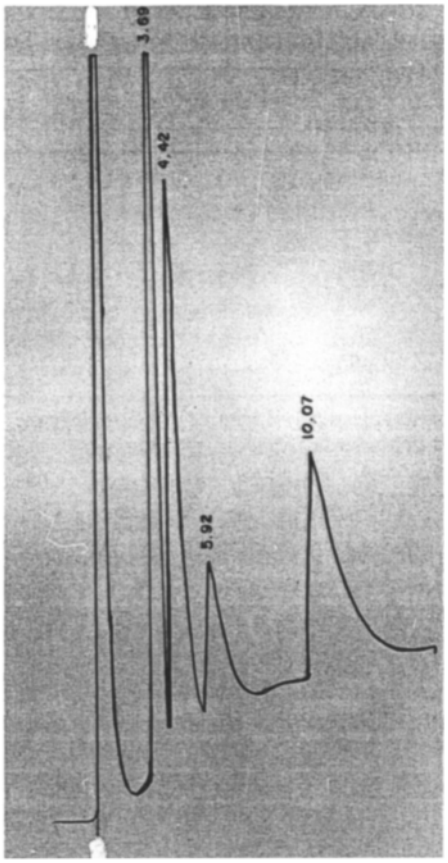

Fig. 3. High-performance liquid chromatography (HPLC) of difructofuranose anhydride (DFA III) and related saccharides. (For run conditions, *see* Materials and Methods; 3.69, 4.42, 5.92, and 10.07 are the respective R_Ts for DFA III, fructose, glucose, and sucrose).

fructose (5.3–5.6 min for the α- and β-anomers of furanose and pyranose rings), glucose (5.9 min), DFA III (13.5 min), and sucrose (19.3 min). GLC-MS displayed a similar fragmentation pattern for the per-silyl DFA III standard and the nonreducing anhydride herein obtained (results not shown). Further data on DFA III were obtained by polarimetry ($[\alpha]_{25} = +128$) and melting point determination (= 161.4–162.4°C range). The literature appoints figures of +135 and 162°C, respectively, which are unlike values of all other difructose anhydrides (I, II, and IV) as well as the related compounds bearing two fructopyranosidic rings, the diheterolevulosans (*17*). Definitive identification of our DFA III product was achieved by HPLC. Care was taken because of the many isomers to be expected from the dehydrating combination of two fructose rings owing to the α- and β-anomericity, the furano-pyrano pair, and C-2 (2′) reactivity towards C-1,3,4,6. DFA III had an R_T of 3.69 min, and those for fructose, glucose, and sucrose were 4.42, 5.92, and 10.07 min, respectively (Fig. 3).

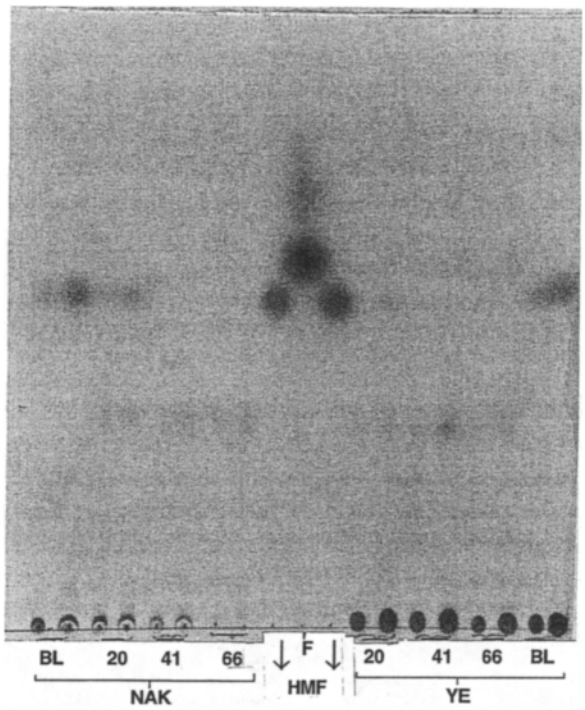

Fig. 4. Thin-layer chromatography (TLC) resolution of hydroxymethylfurfural and its degradation product resulting from the action of isolate "m." 20, 41, 66 = culture time (h) of isolate "m" in Nakamura's (NAK) or yeast extract (YE)-based media at 28°C and 100 rpm. BL = (blank); nonincubated media. Standards (center): HMF = hydroxymethylfurfural; F = mix of furfuraldehyde and furfuryl alcohol (in decreasing order of mobility).

The mould "m" degraded HMF in OPA-hydrolysates of inulin or artificial fructose + HMF mixtures (Fig. 2, left half). The first modification of HMF resulted in a small chromatographic mobility delay and alteration in the reaction with orcinol (pink reaction). HMF was more rapidly degraded (as compared to fructose) in 0.1 g% yeast extract-supplemented medium than in Nakamura's medium. TLC with solvent C allowed distinction between HMF and its immediate derivative (Fig. 4). The derivative was not detected by sulfuric orcinol at the late phase of growth. Its identification is being pursued. There is no possibility of a single de-*O*-methylation of HMF, generating furfural as indicated by the TLC analysis.

Figure 5 summarizes results of the biological activity of our microbial-derived DFA III, using a purified, but not crystallized preparation. If intact liver was affected during the perfusion, the most probable sites of action would be the metabolic pathways responsible for the transformation and oxidation of fructose. The liver is the major site of fructose metabolism, and the isolated perfused organ is an adequate system for measuring metabolic fluxes. One milimolar fructose was infused (repeated experiments)

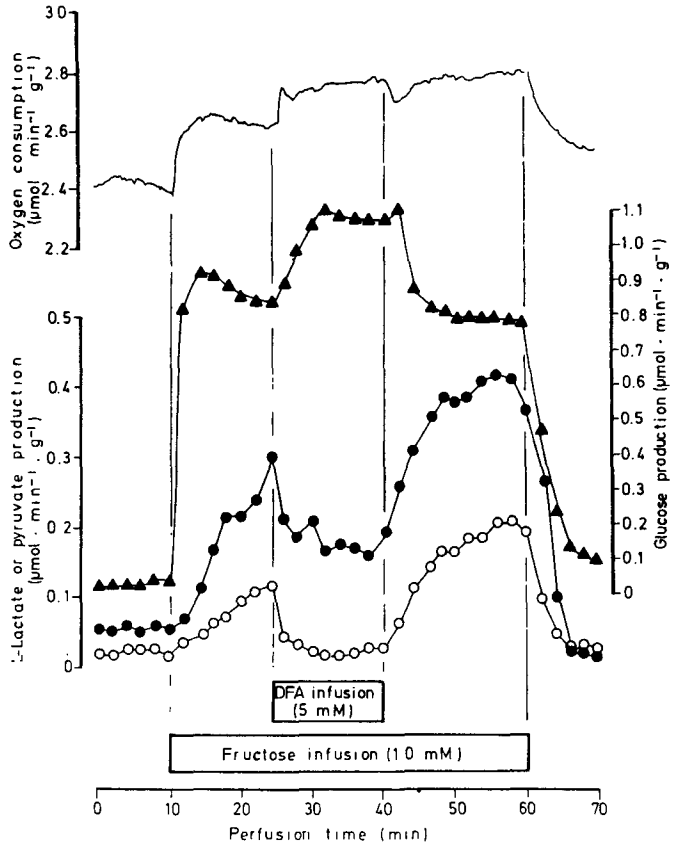

Fig. 5. Difructose anhydride (DFA III) and fructose metabolism: isolated rat liver perfusion. (The liver of a 24-h fasted rat was perfused in an open system as described in Materials and Methods. Rates were expressed as μmol/min/g liver wet wt.). Key: ——= oxygen consumption. ▲——▲ = glucose production. ●——● = L-lactate production. ○——○ = pyruvate production.

at 10–60 min and 5 m*M* DFA III at 25–40 min. Three metabolites produced were measured, glucose, L-lactate, and pyruvate. Oxygen uptake was also measured. Interference by glycogen catabolism was minimal because the rats were starved for 24 h. The onset of fructose infusion increased glucose, lactate, and pyruvate (the latter two because of fructolysis), and oxygen uptake. DFA III inhibited fructolysis, but increased glucose production and oxygen uptake. The negative effect on pyruvate production was particularly pronounced. When the fructose concentration was increased to 5 m*M* in the perfusion solution within a second set of experiments (results not shown), the effect on oxygen uptake was enhanced, but lactate and glucose production were less affected. It should be noted that the action of DFA III was reversible, i.e., when its infusion was stopped, the effects were reversed. Two points were emphasized from these two

experimental variations. First, DFA III is not metabolically inert. It affected at least fructose metabolism. Additional effects on other metabolic pathways are equally possible. Second, DFA probably permeated the cell membrane because the kind of action observed cannot be explained as occurring outside of the cell. For example, inhibition or activation of fructose transport would cause either inhibition or activation of glucose production and fructolysis (*18*). The observed effects on glucose production and fructolysis excluded any disturbance of fructose transport.

Permeation of the cell membrane by DFA III, on the other hand, was quite surprising, because disaccharides, such as sucrose and maltose, are generally not transported across the cell membrane (*18,19*). Owing to its dehydrated structure, DFA III is less polar than other disaccharides and more soluble in organic solvents. TLC inspection after lyophilization of perfusate samples and extraction with 90% methanol showed that the compound was present in the perfusate (38-min sampling time) as an anhydride:fructose proportion resembling that of the preperfusion fluid. On the other hand, no statistical difference was observed in counting of revertants per plate, comparing control and assays using 0.1–10 mg of DFA III in the Ame's test for mutagenicity. In other words, at least within these conditions, DFA III displayed no mutagenicity. Also, incubation of the anhydrodifructosaccharide with isolated rat liver mitochondria using up to 325 nmol FDA/mg mitochondrial protein (total incubation vol = 1.2 mL) did not affect mitochondrial respiration using either succinate or α-ketoglutarate as substrates. DFA III experimentation is being continued with isolated liver for a more accurate evaluation of DFA III biological effect and a finer location of its putative targets at the molecular level. Such experiments are also being performed with HMF, a permanent byproduct of acid processing of inulin despite its reduced generation with optimized hydrolysis kinetics.

CONCLUSIONS

Moderate thermopressurization using phosphoric acid as catalyst (<0.1%; 135°C/2 atm; final pH 3.0–3.5) was shown to be a satisfactory hydrolytic procedure for complete inulin or *dahlia* tuber monomerization to fructose without significant parallel generation of hydroxymethylfurfural. Reduced amounts of difructose anhydride were albeit formed. Thus, although there may be a possible ancillary sweetening role for DFA III (to be more properly designated as β-2,1′:α-2′,3 difructofuranose anhydride), it must be the subject of a deeper investigation of its biological effects. Production of DFA III is attainable with bacterial isolates differing from the classic *Arthrobacter ureafaciens* strain. Work continues in this multifaceted subject, including the question of fungal degradation of HMF.

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