ELSEVIER

Carbohydrate Research 337 (2002) 1185–1191

CARBOHYDRATE RESEARCH

www.elsevier.com/locate/carres

Chemical formation of 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone from D-fructose 1,6-diphosphate☆

Tobias Hauck,[a] Christian Landmann,[a] Thomas Raab,[a] Fredi Brühlmann,[b] Wilfried Schwab[a,*]

[a]*Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany*
[b]*Firmenich SA, Route des Jeunes 1, CH-1211 Geneva, Switzerland*

Received 21 March 2002; accepted 15 May 2002

Abstract

The selective chemical formation of 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (HDF) from D-fructose 1,6-diphosphate in the presence of reduced nicotinamide-adenine-dinucleotides (NAD(P)H) was investigated by means of HPLC–DAD and HPLC–UV–MS/MS. The temperature optimum for HDF formation was 30 °C, whereas the pH value (pH 3–10) and chemical nature of the buffer had no significant influence. A linear correlation of reaction time and D-fructose 1,6-diphosphate concentration with the obtained HDF yield was observed. Proteins appeared to have a stabilizing effect. The NAD(P)H were mandatory, even in the presence of protein, implying a non-enzymatic hydride-transfer to an unknown intermediate which finally leads to the selective formation of HDF. The hydride-transfer was confirmed by the application of selectively pro-4*R* or pro-4*S* deuterium labeled NADH resulting in each case in the formation of HDF exhibiting a deuterium labeling of approx 30% and employment of [4*R*,*S*-$^{2}H_{2}$]-NADH led to a deuterium labeling of approx 66%. The incubation of [1-^{13}C]-D-fructose 1,6-diphosphate with [4*R*,*S*-$^{2}H_{2}$]-NADH revealed that the hydride is transferred to C-5 or C-6 of the D-fructose 1,6-diphosphate skeleton. Thus, a chemical HDF formation from D-fructose 1,6-diphosphate under physiological reaction conditions was shown and for the first time to our knowledge a non-enzymatic hydride-transfer from NADH to a carbohydrate structure was demonstrated.

Keywords: D-Fructose 1,6-diphosphate; Degradation; 4-Hydroxy-2,5-dimethyl-3[2*H*]-furanone; Reduction

1. Introduction

4 - Hydroxy - 2,5 - dimethyl - 3[2*H*] - furanone (HDF, Furaneol®) is an important flavour compound exhibiting an intense caramel-like odour in concentrates and a sweet, strawberry-like odour in dilute solutions. HDF was first discovered in pineapples and has also been isolated from strawberry, raspberry, mangos and other fruits.[1,2] Aside from fruits, HDF has been isolated from insects (e.g., the large wingless cockroach *Eurycotis floridana*), yeasts, bacteria and soy sauce, and it is formed chemically by the reaction of carbohydrates with amines in the so-called Maillard reaction.[2,3] Due to its pleasant taste and low odour threshold (in water: 1–10 μg/mL), it is chemically produced from L-rhamnose on an industrial scale and widely used as a flavouring agent for food and beverages.[4]

It is believed that the naturally occurring furanones in fruits and microorganisms are enzymatically formed. However, no enzyme has been characterized until now. On the basis of chemical and biochemical studies, Pisarnitskii et al. and Zabetakis and Holden postulate 6-deoxysugars as natural precursors, respectively.[5,6] In contrast, studies of Hecquet et al. and Roscher et al. showed the efficient transformation of D-fructose 1,6-diphosphate to HDF by the yeast *Zygosaccharomyces rouxii* and detached strawberry fruits, respectively.[7,8] Also in the Maillard reaction, D-fructose 1,6-diphosphate is rapidly converted into HDF.[9] Since at least one

☆ Dedicated to Professor Peter Schreier on the occasion of his 60th birthday.

* Corresponding author. Tel.: +49(0)-931-8885482; fax: +49(0)-931-8885484
E-mail address: schwab@pzlc.uni-wuerzburg.de (W. Schwab).

0008-6215/02/$ - see front matter
PII: S0008-6215(02)00121-0

reduction step is needed during the transformation of D-fructose 1,6-diphosphate to HDF the involvement of reduced nicotinamide-adenine-dinucleotides (NAD(P)H), cofactors of most dehydrogenases, is assumed in biological systems.[10] Besides, non-enzymatic reductions performed by NAD(P)H as well as NAD-models are well documented and the cofactors also contain free amino functions promoting the Maillard reaction under mild conditions.[11–14]

While investigating the biological transformation of D-fructose 1,6-diphosphate, we observed a significant and selective non-enzymatic formation of HDF from D-fructose 1,6-diphosphate in the presence of NAD(P)H. The influence of various parameters on HDF formation was studied in detail. Experiments using deuterium labeled NADH demonstrated a non-enzymatic hydride-transfer from NADH to a postulated unknown intermediate originating from D-fructose 1,6-diphosphate resulting in the selective formation of HDF.

2. Results and discussion

HDF formation from D-*fructose 1,6-diphosphate and NAD(P)H.*—D-Fructose 1,6-diphosphate was incubated with NAD(P)H and NAD(P) at 30 °C in Tris–Cl buffer (pH 7.5). After 30 h, samples were subjected to solid-phase extraction (SPE) and eluates analyzed by high pressure liquid chromatography with diode array detection (HPLC–DAD). Only one UV absorbing compound was detected exhibiting an UV maximum at 285 nm (Fig. 1). The UV spectrum and retention time were identical to the data obtained for a commercial HDF reference. Further characterization was carried out by HPLC coupled with UV detection and tandem mass spectrometry (HPLC–ESI–UV–MS/MS). A pseudomolecular mass of m/z 170 for the UV absorbing compound due to the acetonitrile adduct of the protonated molecular ion of HDF was determined. The product ion spectrum obtained by low-energy collision-induced dissociation was dominated by the fragment ions m/z 129 caused by the protonated molecular ion of HDF and m/z 101 in consequence of the loss of carbon monoxide (Fig. 1). The experiment was repeated with [1-^{13}C]-D-fructose 1,6-diphosphate and the organic extract obtained by SPE was subjected to gas chromatography–mass spectrometry (GC–MS) analysis. The mass spectrum (Fig. 2) indicates that only singly labeled HDF (molecular ion m/z 129) was formed. Fission of the bonds O–C-5 (or O–C-2) and C-2–C-3 (or C-4–C-5) gives rise to singly labeled fragments at m/z 86, 58 and 44 associated with unlabeled fragments at m/z 85, 57 and 43 with similar intensities and a fission of the C-2–C-3 and the C-4–C-5 bond yields singly labeled ion at m/z 73.[15]

Thus, the combined UV, MS and MS/MS data demonstrated the selective formation of HDF from D-fructose 1,6-diphosphate in the presence of NAD(P)H under physiological conditions. The cofactors turned out to be mandatory, since no formation was observed in their absence. HDF was also formed from D-fructose 1,6-diphosphate in the presence of only NADH or NADPH. However, addition of NAD or NADP increased the production of HDF. The presence of bovine serum albumin (BSA) (pI 4.9) led to significantly increased HDF formation. Amyloglucosidase (pI 3.6) and trypsinogen (pI 9.3) showed similar effects. We postulate a stabilizing effect of the protein on one or more of the intermediates rather than a catalytic impact

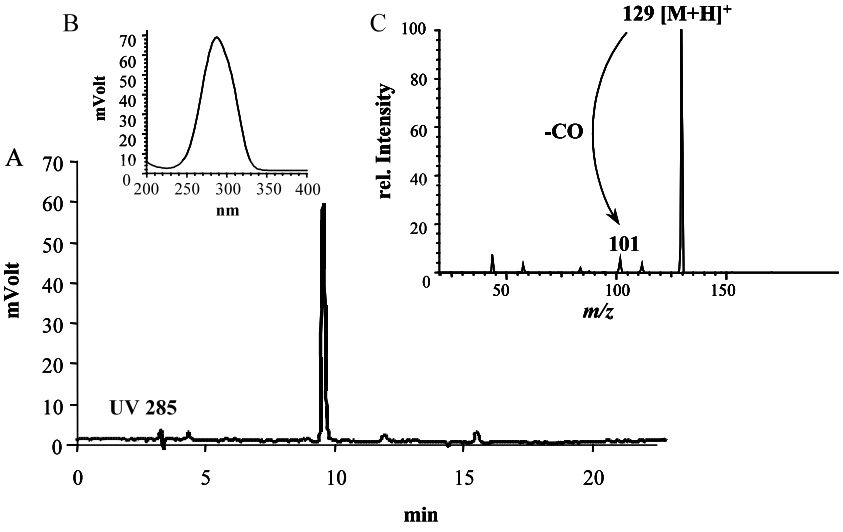


Fig. 1. HPLC–DAD analysis of an extract obtained by solid-phase extraction from an incubation of D-fructose 1,6-diphosphate with NAD(P)H (A), UV spectrum of the compound eluting at 9.5 min (B) and ESI-product ion spectra of the UV absorbing substance (C).

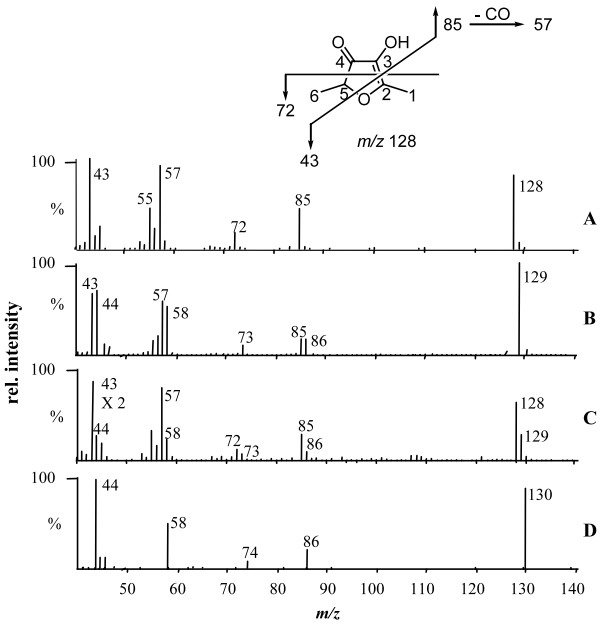


Fig. 2. Mass spectrum of combined HDF isotopomers obtained after incubation of co-factors with unlabeled D-fructose 1,6-diphosphate (A), HDF isotopomers obtained from the incubation of [1-^{13}C]-D-fructose 1,6-diphosphate with the cofactors (B), HDF isotopomers obtained from the incubation of D-fructose 1,6-diphosphate with [4*R*-^{2}H]-NADH (C) and doubly labeled HDF isotopomer obtained from the incubation of [1-^{13}C]-D-fructose 1,6-diphosphate with [4*R*,*S*-$^{2}H_2$]-NADH (D).

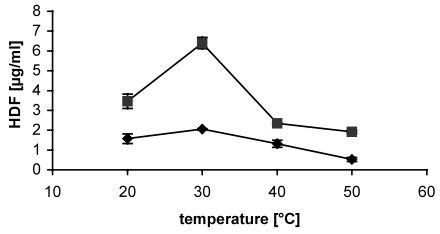


Fig. 3. Effect of the temperature on the formation of HDF in Tris–Cl buffer pH 7.5 (◆) and in Tris–Cl buffer pH 7.5 supplemented with 4.6 mg/mL BSA (■).

because free amino acids were ineffective. However, even in the presence of protein or amino acids the cofactors were needed, indicating a hydride-transfer from the reduced cofactor to the carbohydrate structure. Both phosphate groups appeared to be essential, because neither D-fructose 1-phosphate nor D-fructose 6-phosphate were transformed into the hydroxyfuranone under similar conditions.

Effect of the temperature on HDF formation.—D-Fructose 1,6-diphosphate and cofactors were incubated in Tris–Cl buffer (pH 7.5) at temperatures ranging between 20 °C and 50 °C. Samples supplemented with 4.6 mg/mL BSA each were treated equally. After incubation, solutions were subjected to solid-phase extraction and analyzed by RP18 HPLC with UV detection. Fig. 3 depicts the amounts of HDF obtained with the buffer solutions and the samples containing additional BSA. In both cases, the temperature maximum was 30 °C, whereas the yields obtained with the pure buffer samples were appreciably lower. Obviously, HDF reacts with protein side groups, e.g., ε-amino-groups or even with the amino functions (-NH_2) of the cofactors at higher temperatures. Additional experiments showed, that HDF is stable for 2 days in 20 mM Tris–Cl pH 7.5 even at 40 and 50 °C. Thus, chemical decomposition or evaporation at higher temperatures are unlikely.

Effect of the pH value and the chemical nature of the buffer on the HDF yields.—D-Fructose 1,6-diphosphate and cofactors were incubated in buffer solutions with pH values ranging from pH 3 to pH 10. Two different buffer systems were used for pH values of 5–8 to examine the influence of the chemical nature of the buffer on HDF formation. In the range of pH 3–8 the use of citrate–phosphate buffer and phosphate buffer led to a constant mean HDF production of approx 1.8 µg/mL. When Tris–Cl buffer was used for pH values of 7–10, a slightly increased formation was observed (2.8 µg/mL).

Effect of substrate concentration and reaction time.—The effects of varying amounts of cofactors or D-fructose 1,6-diphosphate on HDF formation are depicted in Fig. 4. HDF yields significantly increased up to cofactor quantities of 0.2 mg/mL and then remained constant at higher concentrations. In contrast, HDF yields depended linearly on the concentration of D-fructose 1,6-diphosphate and no substrate saturation occurred within the chosen range. These observations imply that the rearrangement of D-fructose 1,6-diphosphate to an intermediate which can be reduced by the cofactors is the limiting step of the HDF formation. Incubations for prolonged periods showed that HDF yield increases constantly over a reaction period of 45.5 h.

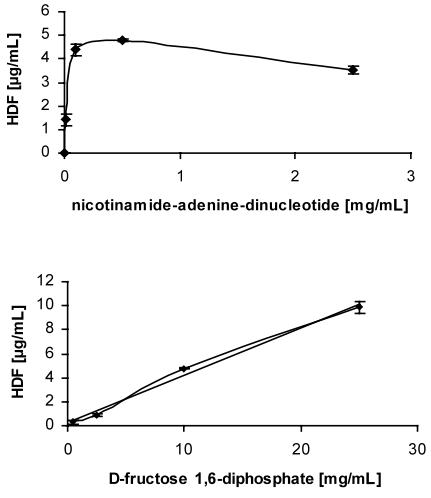
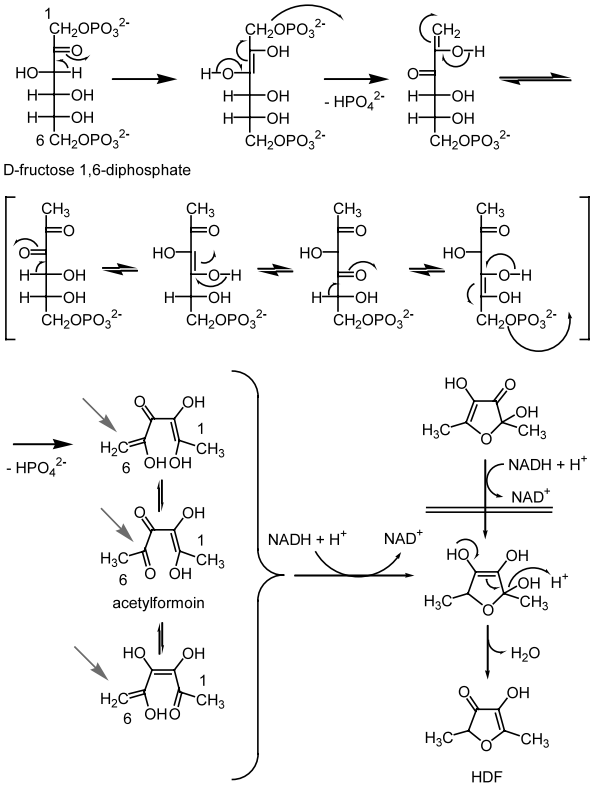


Fig. 4. Effect of cofactor concentration (upper graph) and D-fructose 1,6-diphosphate concentration (lower graph) on the formation of HDF.



Scheme 1. Proposed pathway of HDF formation.

Incubation of D-*fructose-1,6-diphosphate with [4*R-^{2}*H]-NADH, [4*S-^{2}*H]-NADH and [4*R,S-$^2H_2$*]-NADH.*— Since the transformation of D-fructose-1,6-diphosphate into HDF requires a reduction step, a non-enzymatic hydride transfer from reduced NAD to an intermediate originating from D-fructose 1,6-diphosphate was assumed. Several functional groups such as carbonyl groups, azo groups and carbon–carbon double bonds can be reduced non-enzymatically by NADH.[11–13] Aqueous solutions containing deuterium labeled NADH were incubated with D-fructose 1,6-diphosphate under physiological conditions (pH 7.5; 30 °C) and the incorporation of deuterium into the hydroxyfuranone was determined by GC–MS after 30 h of incubation. Deuterium labeled [4*R*-^{2}H]-NADH and [4*S*-^{2}H]-NADH were prepared according to Ottolina and coworkers.[16] [4*R*,*S*-$^2H_2$]-NADH was synthesized by a combination of two published methods.[16,17]

Incorporation of deuterium into HDF was shown by comparison of the intensities of the molecular ions m/z 128 for unlabeled HDF and m/z 129 for the deuterated hydroxyfuranone. The distribution of stable isotopes in HDF obtained after incubation of D-fructose 1,6-diphosphate with deuterated NADH clearly demonstrated the incorporation of one deuterium. After application of stereospecifically deuterated NADH, a deuterium labeling of approx 30% was obtained in case of [4*R*-^{2}H]-NADH and [4*S*-^{2}H]-NADH (Fig. 2). A favoured deuterium transfer from the pro-*R* or the pro-*S* position can therefore be excluded. Application of [4*R*,*S*-$^2H_2$]-NADH confirmed the results obtained by enantioselectively labeled NADH. The mass spectrum of HDF showed a deuterium labeling of approximately 66% which is twice as much as the value determined after the application of singly labeled NADH. Deuterium exchange in the target molecule with water in consequence of the keto-enol tautomerization might account for the remaining 34% which was substantiated by an observed constant decrease of the HDF labeling over a period of 64 h (data not shown). Evaluation of the fragmentation pattern of hydroxyfuranones according to Fay and coworkers shows that the deuterium is apparently incorporated into one of the methyl groups of HDF or in position 5.[15] Since the labeled molecular ion m/z 129 strongly correlates with the fragment ion m/z 73, originating from a fission of the C-2–C-3 and the C-4–C-5 bond deuterium incorporation in the hydroxy-function can be excluded. The deuterium in the hydroxy-group would be rapidly exchanged by protons from the water. Furthermore, the molecular ion m/z 129 correlates with the fragment ions m/z 86, 58 and 44, formed by cleavages of the bonds O–C-5 (or O–C-2) and C-2–C-3 (or C-4–C-5). The labeled fragment ions are associated with unlabeled fragments at m/z 85, 57 and 43 indicating the allocation of the deuterium in one of the methyl groups or in position 2 (or 5). Since the hydroxyfuranone is subjected to keto-enol tautomerization, the proton at C-2 (or C-5) underlies an exchange with the solvent, confirmed by the racemization of enantiomerically pure HDF in water.[18] Thus, the deuterium might be located in one of the methyl groups which is also confirmed by the similar HDF fragmentation pattern in the mass spectra obtained from samples containing 1-^{13}C-D-fructose 1,6-diphosphate or deuterated NADH (Fig. 2).

On the basis of our results, we propose a reaction mechanism shown in Scheme 1. Since a remarkable portion of D-fructose 1,6-diphosphate is available in the reactive carbonyl form (1.7%),[19] an initial enolization via an enediol structure leads to the elimination of inorganic phosphate. Additional enediol formation and phosphate elimination yields the isomeric structures of 3,4,5-trihydroxy-3,5-hexadien-2-one (acetylformoin). HDF can be formed after the reduction of the carbon–carbon double bond or the carbonyl-function in position 2 (or 5) by a hydride transfer from NADH to C-1 (C-6) or C-2 (C-5), cyclization and dehydration. However, incubations under identical conditions with acetylformoin instead of the sugar phosphate did not produce HDF. This might be explained by the fact, that in aqueous solutions acetylformoin is available only in the cyclic tautomeric form.[20] Experiments with NADH mimics and carbonyls have shown that after formation of a covalent bond an intramolecular hydride transfer

occurs.[21] Accordingly, we assume an enamine intermediate formed from NADH and D-fructose 1,6-diphosphate inhibiting cyclization of the pyranose ring and thus enhancing reactivity and promoting phosphate elimination. After an intramolecular hydride transfer, NAD is released and HDF is formed. The proposal is supported by the fact that acetylformoin in the cyclic form and NADH do not produce HDF.

*Incubation of [1-^{13}C]-D-fructose 1,6-diphosphate with [4*R*,S-$^{2}H_2$]-NADH.*—Doubly labeled [4*R*,*S*-$^{2}H_2$]-NADH was incubated under standard conditions with [1-^{13}C]-D-fructose 1,6-diphosphate to localize the deuterium label in the target molecule. After 40 h, samples were worked-up and analyzed by GC–MS. HDF fragment ions m/z 130 and m/z 74 indicate the presents of two labels (^{13}C and ^{2}H) in the molecule (Fig. 2). However, fragment ions m/z 86, m/z 58 and m/z 44 carry only one label. This fragmentation pattern can only be explained by the assumption that the ^{2}H label is attached either to carbon C-5 or C-6 when the numbering starts with ^{13}C in position 1. This implies, that the hydride is transferred to C-5 or C-6 of the D-fructose 1,6-diphosphate skeleton as shown in Scheme 1.

3. Conclusion

We showed for the first time that HDF can be formed under physiological conditions (pH 7.5, 30 °C) from D-fructose 1,6-diphosphate by a non-enzymatic reduction with NAD(P)H. Although the chemical yield is rather low (approx 0.1%), the experiments with deuterium labeled NADH clearly demonstrated the hydrid-transfer from NADH to C-5 or C-6 of the carbohydrate structure. However, the observed non-enzymatic formation of HDF from D-fructose 1,6-diphosphate and NADH cannot account for the naturally detected concentration of HDF, e.g., 10 mg/kg strawberry fresh weight. Natural D-fructose 1,6-diphosphate concentrations of 0.02–0.05 mg/g fruit fresh weight have been reported which are by a factor of at least 200 lower than the concentrations we used in our experiments.[22,23] Thus, we assume an additional enzymatic formation pathway in biological systems.

4. Experimental

General methods.—HPLC analysis with UV-detection was performed using an HPLC system equipped with a Spark Holland Basic marathon autosampler (Spark Holland, Emmen, The Netherlands) connected to a Knauer Maxistar pump and Knauer variable wavelength monitor (Knauer, Berlin, Germany). Knauer Eurochrom 2000 software was used for data acquisition and evaluation. For HPLC analysis with diode array detection (DAD), a Hewlett–Packard (Waldbronn, Germany) 1100 HPLC gradient pump and a Hewlett–Packard 1100 photodiode array detector were used including Hewlett–Packard chemstation software for data acquisition and evaluation. A Eurospher 100-C18 column (length 250 mm × 4 mm i.d., particel size 5 μm) (Knauer) was employed. A binary gradient starting from 95% (A: 0.05% formic acid in water) and 5% (B: acetonitrile) to 80% A within 10 min then to 0% A in 30 min was used at a flow rate of 1 mL/min. Injection volume was 20 μL. The HDF yields were quantified using a standard curve of commercial HDF. HDF concentration was calculated as mean value of duplicate analysis. High pressure liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) was performed utilizing a TSQ 7000 tandem mass spectrometer system equipped with an electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany) and an Applied Biosystems (BAI, Bensheim, Germany) 140b pump. Data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany) using Finnigan MAT ICIS 8.1 software. HPLC separation with MS detection was carried out on a XTerra MS C18 column (length 150 mm × 2.1 mm i.d., particle size 3.5 μm) (Waters, Milford, MA, USA) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. HPLC was programmed as described above except of using a flow rate of 200 μL/min. The injection volume was 5 μL and mass spectra were acquired in the positive ion mode. For pneumatic assisted ESI, the spray voltage was set to 3.5 kV, the temperature of the heated capillary was 210 °C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 units). Product ion scanning was performed at a collision gas pressure of 2.0 mtorr and a collision energy of 15 eV with a total scan duration of 1.0 s for a single spectrum. Capillary GC–MS analysis was performed with a Fisons Instruments (Fisons, Engelsbach, Germany) GC 8000 Series coupled to a Fisons Instruments MD800 quadrupol mass detector fitted with a split-injector (1:20) at 230 °C. A J & W DB-Wax fused silica capillary column (30 m × 0.25 mm i.d.; df = 0.25 μm) (J & W, Folsom, CA, USA), which was programmed from 50 °C for 3 min then to 220 °C (for 10 min) with a temperature increase of 4 °C/min, was employed with 2 mL/min flow rate of helium gas. The software Xcalibur for windows was used for data acquisition. Significant MS operating parameters were: ionization voltage, 70 eV (electron impact ionization); ion source temperature, 220 °C; interface temperature, 250 °C. UV spectra were recorded on a Shimadzu (Duisburg, Germany) UV2101PC spectrophotometer.

Reagents.—Chemicals, salts, and solvents of high purity were obtained from Fluka (Deisenhofen, Germany), Sigma (Deisenhofen) and Aldrich (Deisen-

hofen). Solvents were redistilled prior to use. Water of HPLC gradient grade was from Merck (Darmstadt, Germany) and acetonitrile of HPLC gradient grade was from Fisher (Loughborough, UK). Glucose dehydrogenase (from *Thermoplasma acidiphilum*, recombinant; expressed in *E. coli*), formate dehydrogenase (from yeast) and L-glutamic dehydrogenase (from bovine liver) were obtained from Sigma. D-[1-^{2}H]-Glucose (98% ^{2}H) and [1-^{13}C]-D-fructose 1,6-diphosphate (sodium salt, MW 506.7) were purchased from Omicron (South Bend, IN, USA). [$^2H_2$]-Formic acid was obtained from Aldrich. 3,4,5-Trihydroxy-3,5-hexadien-2-one (acetylformoin) was kindly supplied from Firmenich.

General procedure.—Aliquots (2 mL) of 20 mM buffer solution (phosphate or Tris–Cl) were supplemented with substrate (1–50 mg D-fructose 1,6-diphosphate, D-fructose 6-phosphate or D-fructose 1-phosphate) or 50 mg 1-^{13}C-D-fructose 1,6-diphosphate or 7 mg acetylformoin. Equal amounts of NAD, NADH, NADP and NADPH (0–5 mg each) were added. The solutions were kept with gentle agitation for various periods at 30 °C.

Analysis.—The samples were subjected to solid-phase extraction (SPE) using RP18 cartridges (Supelco, 500 mg/3 mL), preconditioned with MeOH (6 mL) and water (6 mL). After application the cartridges were rinsed with 1 mL water and eluted with 3 mL diethyl ether. The water remaining in the diethyl ether extract was removed by freezing (-18 °C). Water (200 μL) was added to the organic phase and the diethyl ether was removed by a stream of nitrogen. The aqueous phase was analyzed by HPLC with UV detection at 285 nm or diode array detection.

Determination of the time dependency of the HDF formation.—Tris–Cl buffer (20 mL, pH 7.5) was incubated with 200 mg D-fructose 1,6-diphosphate and 10 mg of each cofactor at 30 °C. After 9.5, 21.5, 33.5 and 45.5 h, 2 mL aliquots were withdrawn and the HDF concentration was determined after SPE by HPLC–UV.

Effect of the temperature on HDF formation.—Aliquots (2 mL) of 20 mM Tris–Cl buffer (pH 7.5) were supplemented with 20 mg D-fructose 1,6-diphosphate and 1 mg of each cofactor. Samples were kept for 30 h at temperatures in the range of 20 to 50 °C. Samples containing additionally 4.6 mg/mL BSA were treated in the same way. After SPE the HDF concentration was determined by HPLC–UV.

Effect of the pH value on HDF formation.—Aliquots (2 mL) of 20 mM buffer solutions in a pH range from pH 3 to pH 10 containing 20 mg D-fructose 1,6-diphosphate and 1 mg of each cofactor were kept for 43 h at 30 °C. Diluted McIllvaine citrate–phosphate buffer was used in the range of pH 3 to pH 6, phosphate buffer in the range of pH 5 to pH 8 and Tris–Cl buffer in the range of pH 7 to pH 10. Samples were analyzed by SPE followed by HPLC–UV.

Effect of protein on HDF formation.—Four samples consisting of 20 mM Tris–Cl buffer (2 mL, pH 7.5), 20 mg D-fructose 1,6-diphosphate and 9.2 mg BSA were prepared. Additionally 1 mg of each cofactor (NAD, NADH, NADP, NADPH) was added to two of the solutions. Samples were kept for 30 h at 30 °C. HDF concentrations were determined by HPLC–UV. Similar experiments were performed with amyloglucosidase and trypsinogen.

*Preparation of [4*R-^{2}*H]-NADH.*—A solution (6 mL) containing 0.05 M sodium carbonate, 0.05 M [$^2H_2$]-formic acid, 0.015 M NAD and 20 U of formate dehydrogenase was adjusted to pH 8.5 and kept at room temperature. The course of NAD reduction was monitored by UV spectroscopy at 340 nm. After 3 h, the reaction was completed. The solution was diluted with water to 15 mL and loaded onto a DEAE Sepharose column (2 × 14 cm; Pharmacia) equilibrated with water. The column was eluted with a linear ammonium bicarbonate gradient (0–0.4 M, 300 mL) at a flow rate of 9 mL/h and 4 °C. Absorbance of the fractions (4.5 mL) was measured at 260 nm. NAD and NADH were identified by their characteristic UV spectra and [4*R*-^{2}H]-NADH was recovered by lyophilization of the combined fractions. Incorporation of deuterium was confirmed by means of LC–MS analysis. Isotopically pure deuterium labeled NADH showing a m/z 667 $[M+H]^+$ was obtained.

*Preparation of [4*S-^{2}*H]-NADH.*—A solution (6 mL) containing 0.05 M sodium bicarbonate, 0.03 M D-[1-^{2}H]-glucose, 0.015 M NAD and 35 U of glucose dehydrogenase was adjusted to pH 7.5 and kept at 30 °C overnight. The course of NAD reduction was monitored by UV spectroscopy at 340 nm. The solution was processed as described above and LC–MS analysis led to identical results (m/z 667 $[M+H]^+$).

*Preparation of [4*R,S-$^2H_2$*]-NADH.*—[4*R*-^{2}H]-NADH was prepared as described above. After the reduction was complete, the formate dehydrogenase was inactivated by the addition of acetonitrile (15 mL). Acetonitrile was removed by rotatory evaporation at 40 °C and the water by lyophilization. The white residue was dissolved in water (6 mL) and the pH adjusted to 7.6 with 0.1 N HCl. [4*R*-^{2}H]-NADH was converted to [4-^{2}H]-NAD^+ by the addition of 55 U L-glutamic dehydrogenase, α-ketoglutarate (20 mM final concentration) and 50 μL of a 1 M NH_4Cl stock solution. After completion of the oxidation, the enzyme was again inactivated by the addition of acetonitrile (15 mL) and proceeded as described above. [4*R*,*S*-$^2H_2$]-NADH was prepared by reduction of [4-^{2}H]-NAD^+ with formate dehydrogenase in the presence of deuterated formic acid in a manner similar to that described for [4*R*-^{2}H]-NADH. The doubly deuterated cofactor was isolated by anion-exchange chromatography on DEAE as described above and LC–MS analysis led to a labeling

pattern of 95.9% [4*R*,*S*-$^2H_2$]-NADH (*m*/*z* 668) and 4.1% of the corresponding singly labeled compound (*m*/*z* 667).

Incubations with deuterium labeled NADH and determination of deuterium incorporation.—Aliquots (2 mL) of 20 mM Tris–Cl (pH 7.5) containing 50 mg D-fructose 1,6-diphosphate and approx 2 mg [4*R*-^{2}H]-NADH, [4*S*-^{2}H]-NADH or [4*R*,*S*-$^2H_2$]-NADH, respectively, were gently shaken at 30 °C for 30 h. HDF was separated by SPE and analyzed by GC–MS. Incorporation of deuterium into HDF was shown by comparison of the intensities of the molecular ions *m*/*z* 128 for unlabeled HDF, *m*/*z* 129 for the monodeuterated hydroxyfuranone and *m*/*z* 130 for doubly labeled HDF. The intensity of the molecular ion of labeled HDF was calculated as percentage of the sum of the intensities of the molecular ions of unlabeled and labeled HDF.

Acknowledgements

Financial support from Firmenich is gratefully acknowledged.

References

1. Rodin J. O.; Himel C. M.; Silverstein R. M.; Leeper R. W.; Gortner W. A. *J. Food Sci.* **1965**, *30*, 280–285.
2. Schwab W.; Roscher R. *Recent Res. Dev. Phytochem.* **1997**, *1*, 643–673.
3. Nunomura N.; Sasaki M.; Yokotsuka T. *Agric. Biol. Chem.* **1980**, *44*, 339–351.
4. Larsen M.; Poll L. *Z. Lebensm.-Unters. Forsch.* **1992**, *195*, 120–123.
5. Pisarnitskii A. F.; Demechenko A. G.; Egorov I. A.; Gvelesiani R. K. *Prikl. Biokhim. Mikrobiol.* **1992**, *28*, 123–127.
6. Zabetakis I.; Holden M. A. *Plant Cell, Tissue Organ Cult.* **1996**, *45*, 25–29.
7. Hecquet L.; Sancelme M.; Bolte J.; Demuynck C. *J. Agric. Food Chem.* **1996**, *44*, 1357–1360.
8. Roscher R.; Bringmann G.; Schreier P.; Schwab W. *J. Agric. Food Chem.* **1998**, *46*, 1488–1493.
9. Schieberle P. *ACS Symp. Ser.* **1992**, *490*, 164–174.
10. Dahlen T.; Hauck T.; Wein M.; Schwab W. *J. Biosci. Bioeng.* **2001**, *91*, 352–358.
11. Miwa I.; Okuda J. *Biochem. Pharmacol.* **1982**, *31*, 921–925.
12. Nam S.; Renganathan V. *Chemosphere* **1999**, *40*, 351–357.
13. Wang H.; Liu Y.; Guo Q. *Lanzhou Daxue Xuebao, Ziran Kexueban* **1999**, *35*, 64–70.
14. Njoroge F. G.; Monnier V. M. *Prog. Clin. Biol. Res.* **1989**, 85–107.
15. Fay L. B.; Huynh-Ba T.; Blank I. *J. Agric. Food Chem.* **1997**, *45*, 4057–4064.
16. Ottolina G.; Riva S.; Carrea G.; Danieli B.; Buckmann A. F. *Biochim. Biophys. Acta* **1989**, *998*, 173–178.
17. Kurz L. C.; Frieden C. *Biochemistry* **1977**, *16*, 5207–5216.
18. Bruche G.; Schmarr H. G.; Bauer A.; Mosandl A.; Rapp A.; Engel L. *Z. Lebensm.-Unters. Forsch.* **1991**, *193*, 115–118.
19. Benkovic S. J. *Methods Enzymol.* **1979**, *63*, 370–379.
20. Engel W.; Hofmann T.; Schieberle P. *Eur. Food Res. Technol.* **2001**, *213*, 104–106.
21. Meyers A. I.; Brown J. D. *J. Am. Chem. Soc.* **1987**, *109*, 3155–3156.
22. Salminen S. O.; Young R. E. *Plant Physiol.* **1975**, *55*, 45–50.
23. Parekh L. J.; Sakariah K. K.; Shah V. J. *Enzymol. Acta Biocatal.* **1970**, *38*, 23–28.