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Note

Formation of 2-(2-furoyl)-4(5)-(2-furyl)-1*H*-imidazole in the Maillard reaction

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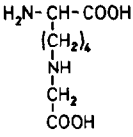
It has long been known that reactions occur between proteins and glucose[1] *in vivo* which lead to the formation of 1-amino-1-deoxyfructose, usually involving the ε-amino group of a lysine residue. Similar reactions (Maillard or browning), which lead to degradation of sugars, occur when foods are heated or stored[2]. Reducing sugars are converted, *inter alia*, into 1-amino-1-deoxyfructoses in the presence of amino acids or proteins[3], and other degradation products may then arise in a series of secondary reactions[4]. These compounds can be detected in heated foods[5] and the question arises as to whether they are formed also *in vivo*.



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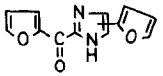

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The identification of two compounds isolated from *in-vivo* material (carboxymethyl-lysine, **1**) or from model systems of proteins and glucose (FFI, **2**) has been reported. The formation of **1** can be explained *via* oxidative cleavage of lysinodeoxyfructose[6]. 2-(2-Furoyl)-4(5)-(2-furyl)-1*H*-imidazole (FFI, **2**) was isolated[7] from reaction mixtures of polylysine and bovine serum albumin with glucose. After acid hydrolysis of the proteins, FFI was separated from the solution at pH 11. The excitation fluorescence spectrum of FFI was largely consistent with those obtained from the products of reaction of bovine serum albumin or polylysine with glycose. Hence, it was suspected that a product was formed from which FFI was released by the action of acid (110°, 6M HCl, 10 h). The imidazolium compound **5** was assumed

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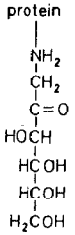

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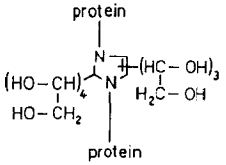

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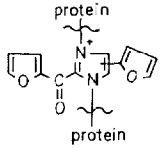


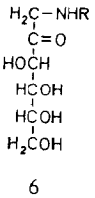
5

to be the precursor (**3**→**4**→**5**) from which FFI is formed after acid hydrolysis.

We have investigated this reaction step using as a model compound propyl-FFI **12**, obtained by reaction of 2-furylglyoxal hydrate with propylamine. However, treatment of **12** with hydrochloric acid under conditions corresponding to those of protein hydrolysis did not give any FFI. Likewise, treatment of the reaction mixture of propylamine and furylglyoxal with acid gave no FFI. Thus, the imidazolium compound **5** cannot be the precursor of FFI.

When the experiment of Pongor *et al.*[7] was repeated with propylamine instead of protein, FFI could be detected after treatment of the reaction mixture with acid when the pH of the solution was adjusted to 11 with ammonia. If sodium hydroxide or triethylamine was added instead of ammonia, FFI could not be detected. Treatment of the glucose–propylamine reaction solution with acid was necessary in order to produce FFI subsequently with ammonia.

It has long been known that furan derivatives of the type **7** and pyridones of structure **8** can be formed from 1-amino-1-deoxyketoses **6** by the action of acid[8]. The amount of 1-amino-1-deoxyketoses in foods and also *in vivo* can be determined by means of these compounds[9]. The furan **7a**, synthesized by treatment of 1-deoxy-1-propylamino-D-fructose with acid, was converted into FFI and propyl-FFI on treatment with ammonia. The ratio of the two products depended on the amount of ammonia added. Presumably, at pH ≥8, **7a** tautomerizes to give **9**, which is oxidized to the furoyl-imine **10** and hence to furan-2-carboxylic acid **11**. In the intermediates **9** and **10**, the propylamine can be readily exchanged by ammonia, and condensation reactions then yield propyl-FFI (**12**) and FFI (**2**). Treatment of **7a** with ammonia at pH ≤7 did not give **2** and **12**.



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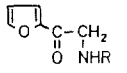
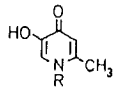


7a R = Pr
7b R = lysyl



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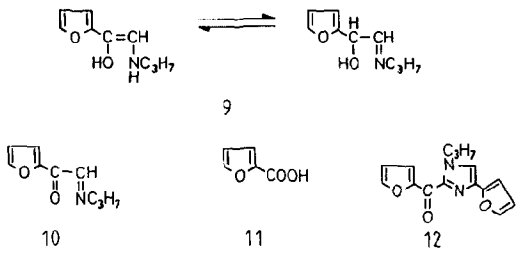

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10 11 12

Thus, it is assumed that the reaction of polylysine and bovine serum albumin with glucose leads, *inter alia*, to formation of 1-amino-1-deoxyketoses of the type **3**. Reaction with acid then gives **7b**, which is readily oxidized at pH 11, and the excess of ammonia then displaces the lysine and FFI is formed.

Brief reaction of lactate dehydrogenase with furylglyoxal and D-glucose 6-phosphate generated excitation fluorescence spectra with excitation and emission in the same wavelength region as in the fluorescent spectrum of propyl-FFI (see Fig. 1). Section A of Fig. 2 shows the reduction of the activity of lactate dehydrogenase and the generation of fluorescence spectra on incubation with D-glucose, D-glucose 6-phosphate, or furylglyoxal. The results suggest that ε-amino groups of lysine residues in the lactate dehydrogenase have reacted with D-glucose, D-glucose 6-phosphate, and furylglyoxal to form a protein-bound FFI. The ammonia required might have been released, for example, by hydrolysis of the amide group in asparagine or glutamine. Further experiments are necessary to establish the extent to which furylglyoxal and similar substances are formed from glucose *via* the Maillard reaction.

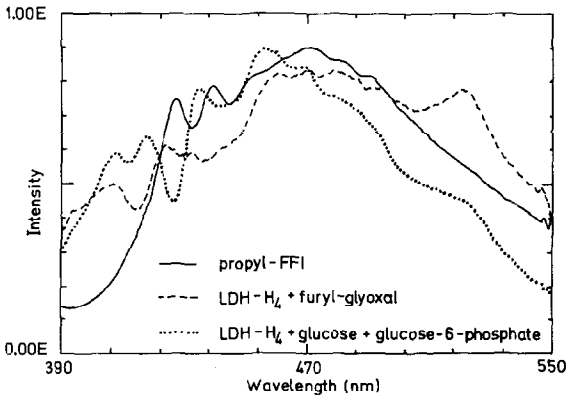

Fig. 1. Fluorescence properties of propyl-FFI (20 μg in 1 mL of 67mM potassium dihydrogen phosphate, pH 7.2) represented by ——; incubation 2 (see Experimental) for 47 days at 37°, ·····; incubation 5 for 22 days at 37°, – – – – –. Excitation at 370 nm, 25°, band widths: excitation, 4.5 nm; emission, 2.25 nm. In order to permit a better comparison, the maxima were arbitrarily adjusted to about the same intensity.

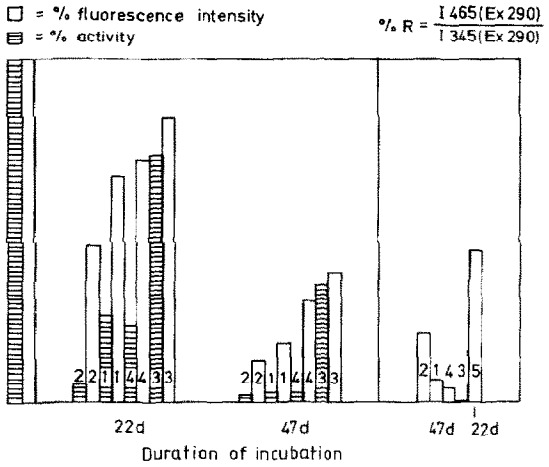

Fig. 2. A, Fluorescence intensity and lactate dehydrogenase activity [measured on a solution of 20 μL of 17mM NADH, 20 μL of 31mM pyruvate, *x* μL of incubates *1–4*, and 960 − *x* μL of 67mM potassium dihydrogen phosphate (pH 7.2)]; B, ratio of extrinsic and intrinsic fluorescence properties for incubates *1–5*: *1* LDH-H_4 + D-glucose; *2*, LDH-H_4 + D-glucose + D-glucose 6-phosphate; *3*, LDH-H_4; *4* acetamidated LDH-H_4 + D-glucose; *5*, LDH-H_4 + furylglyoxal (see Experimental for details).

EXPERIMENTAL

General methods. — Melting points were determined with a Büchi 510 apparatus and are uncorrected. I.r. spectra were recorded for KBr discs with a Perkin–Elmer 197 spectrometer. N.m.r. spectra (internal Me_4Si) were recorded with a Varian A-60 spectrometer. Mass spectra were produced with a Varian MAT CH7 spectrometer equipped with a probe inlet. Silica Gel 60 F_{254} (Merck, 5554 and 5717) was used for t.l.c., and Silica Gel 60 (Merck, 9385) for column chromatography.

H.p.l.c. was performed on a Merck–Hitachi system (pump L-6000, u.v. detector L-4000, and chromato integrator D-2000) with a Nucleosil (Bischoff, 25462835) reversed phase C-18 column (250 × 4.6 mm) and methanol–water (65:35).

G.l.c. was performed with a Perkin–Elmer 8320 instrument equipped with a flame-ionisation detector and a quartz capillary column (25 m × 0.25 mm i.d., permaphase, Perkin–Elmer 698345) coated with dimethylsilicone; injection and detection ports at 280°; temperature programme 100°→200° at 6°/min (propyl-FFI), and 60°→120° at 6°/min then 120°→260° at 10°/min (silylated furan-2-carboxylic acid).

Fluorescence spectra were obtained with a Spex Industries instrument (fluorolog 2) at 25° with band widths of 4.5 nm (excitation) and 2.25 nm (emission).

Concentration centrifugation was done with a Grace & Co, Amicon Division instrument (centricon).

*2-(2-Furoyl)-4(5)-(2-furyl)-1*H*-imidazole (FFI,* **2***).* — Compound **2**, prepared from 2-furylglyoxal hydrate[10] as described by Pongor *et al.*[7], had m.p. 179–180° (lit.[7] m.p. 176–177°); ν_{max} 3225, 1630, 1580 cm^{-1}. The ^{1}H-n.m.r., u.v. and mass-spectral data were identical to those reported[7].

2-(2-Furoyl)-4-(2-furyl)-1-propylimidazole (propyl-FFI, **12***).* — To a solution of 2-furylglyoxal hydrate[10] (710 mg, 5 mmol) in ethanol (10 mL) and water (2 mL) were added propylamine (0.5 mL) and 6M ammonia (1 mL) in small portions. The mixture was stored for 3 h at room temperature, the solvents were evaporated, and unreacted propylamine and ammonia were removed under diminished pressure. Column chromatography (2:8 ethyl acetate–hexane) of the syrupy residue gave a fraction (2 fluorescent spots at 360 nm in t.l.c. with 2:8 ethyl acetate–hexane) corresponding to compounds **2** and **12**. Preparative t.l.c. (3:7 ethyl acetate–hexane) gave **2**, R_F 0.2, and **12**, R_F 0.5. Compound **12** (115 mg, 17%), extracted with ethyl acetate, had m.p. 58–59°; ν_{max} 3090, 2950, and 1630 cm^{-1}. ^{1}H-N.m.r. data ($CDCl_3$): δ 0.97 (t, 3 H, *J* 7 Hz), 1.90 (m, 2 H), 4.50 (t, 2 H, *J* 7 Hz), 6.55 (dd, 1 H, *J* 3.5 and 1.5 Hz), 6.68 (dd, 1 H, *J* 3.5 and 1.5 Hz), 6.83 (d, 1 H, *J* 3.5 Hz), 7.47 (s, 1 H), 7.52 (d, 1 H, *J* 1.5 Hz), 7.82 (d, 1 H, *J* 1.5 Hz), 8.32 (d, 1 H, *J* 3.5 Hz). Mass spectrum: *m/z* 270 (66%, M^+), 227 (15), 160 (22), 109 (24), 95 (89), 84 (92), 82 (100), 69 (44).

Anal. Calc. for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.37. Found: C, 66.65; H, 5.39; N, 10.21.

2-(2-Furyl)-2-oxoethyl(propyl)ammonium oxalate (salt of **7a***).* — 1-Deoxy-1-propylamino-D-fructose oxalate[11] (6.22 g, 20 mmol) was heated in 6M hydrochloric acid (100 mL) for 12 h at 100–110°. The solution was then cooled, filtered, and concentrated under diminished pressure. Column chromatography (6:4 ethyl acetate–methanol) of the residue gave **7a**, R_F 0.55 (t.l.c.), which gave a yellow spot with ninhydrin and a red spot with alkaline triphenyltetrazolium chloride. Oxalic acid (300 mg) was added to the appropriate fractions, the solvents were removed under reduced pressure, and the residue was recrystallized from ethanol to give **7a** oxalate (570 mg, 11%), m.p. 214° (dec.); ν_{max} 3450, 1705, 1595 cm^{-1}. ^{1}H-N.m.r. data (CD_3OD): δ 1.08 (t, 3 H, *J* 3.5 Hz), 1.83 (m, 2 H), 3.20 (t, 2 H, *J* 3.5 Hz), 4.66 (s, 2 H), 6.82 (dd, 1 H, *J* 1.5 and 3.5 Hz), 7.66 (d, 1 H, *J* 1.5 Hz), 7.98 (d, 1 H, *J* 3.5 Hz). Mass spectrum: *m/z* 167 (10%, M^+), 110 (34), 95 (42), 81 (46), 72 (100), 58 (52).

Anal. Calc. for $C_{11}H_{15}NO_6$: C, 51.36; H, 5.88; N, 5.44. Found: C, 51.34; H, 5.90; N, 5.47.

The pH of a solution of **7a** (20 mg) in methanol (0.7 mL) and water (0.3 mL) was adjusted to 8 with 0.1M ammonia. After a few minutes, **2** (R_F 0.45) and **7a** (R_F 0.7) were detectable by t.l.c. (3:7 ethyl acetate–hexane). In h.p.l.c., **2** was eluted after 7.42 min and **7a** after 18.09 min in the ratio 3:1 (flow rate, 1 mL/min; and absorbance at 285 nm).

G.l.c. of the silylated products (0.2 mL of pyridine, 0.2 mL of chlorotrimethylsilane, and 0.1 mL of hexamethyldisilazane overnight at room temperature)

gave a peak (T 10.59 min) for trimethylsilylated furan-2-carboxylic acid.

At pH 7 (adjusted with ammonia), 7.8 and 10 (adjusted with sodium hydroxide and sodium carbonate), **2** and **7a** were not detectable.

Incubation of lactate dehydrogenase (LDH-H_4) with D-*glucose,* D-*glucose 6-phosphate, and 2-furylglyoxal.* — LDH-H_4 was isolated from pig heart and purified as described by Pfleiderer and Jeckel[12]. Incubations were performed in 67mM potassium dihydrogen phosphate (pH 7.2) protected with 5mM sodium azide from microbial degradation and containing *1*, LDH-H_4 (2 mg/mL) and 100mM D-glucose; *2*, LDH-H_4 (2 mg/mL), 100mM D-glucose, and 50mM D-glucose 6-phosphate; *3*, LDH-H_4 (2 mg/mL); *4*, acetamidated LDH-H_4 (2 mg/mL; prepared according to the method of Pinner[13]) and 100mM D-glucose; *5*, LDH-H_4 (4 mg/mL) and 5mM furylglyoxal hydrate. The solutions *1–5* were kept for up to 47 days at 37°. Spectral data were obtained after the removal of denatured LDH-H_4 by centrifugation. Low-molecular-weight compounds were separated on a G-25 column (Pharmacia-LKB) and concentration was performed by membrane centrifugation.

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