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Aging of proteins: Isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose

(Maillard reaction/nonenzymatic glycosylation/nonenzymatic browning/furoyl furanyl imidazole)

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ABSTRACT **Proteins exposed to glucose over long periods are known to undergo physicochemical changes including crosslinking and formation of brown fluorescent pigments of poorly characterized structure. Acid hydrolysis of both browned poly(L-lysine) and browned bovine serum albumin is found to release a major fluorescent chromophore, which after alkalinization is extractable into organic solvents and which can be purified by silica gel chromatography. The fluorescence properties of this compound very closely resemble those of the bulk browned polypeptides. By NMR, mass spectroscopy, and chemical derivatization, this compound is assigned the structure 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (FFI). Confirmation was obtained by independent chemical synthesis from furylglyoxal and ammonia. The incorporation of two peptide-derived amine nitrogens and two glucose residues in FFI strongly suggests that peptide-bound FFI precursors are implicated in the crosslinking of proteins by glucose *in vivo*. This reaction has potential implications in the understanding of glucose-mediated protein modifications and their role in the complications of diabetes and aging.**

The appearance of brown pigments during the cooking of food is a universally noted phenomenon. The chemical basis for this process was first outlined by Maillard in 1912, when he observed that glucose or other reducing sugars react with amino acids to form adducts that undergo a series of dehydrations and rearrangements to form stable brown pigments (1). In the ensuing years, food chemists have studied the Maillard reaction in detail. Stored and heat-treated foods undergo nonenzymatic browning that crosslinks proteins and decreases their bioavailability (2). The chemical structures of brown crosslinking pigments have not been elucidated, although they possess characteristic spectra and fluorescent properties.

In recent years it has become evident that the Maillard reaction also occurs *in vivo*. This was first substantiated by the finding that glucose was attached via an Amadori rearrangement to the amino-terminal of the α-chain of hemoglobin (3, 4). Subsequently, glucose–protein adducts have been found *in vivo* for a number of proteins (5). Brown pigments with spectral and fluorescent properties similar to those of late-stage Maillard products have also been observed *in vivo* in association with several long-lived proteins—e.g., lens proteins and collagen—from aged individuals (6, 7). In fact, an age-related linear increase in pigment was observed in human dura collagen between the ages of 20 to 90 years (7).

Besides imparting a color to these proteins, the pigments, by their crosslinking nature, could modify these long-lived proteins and account for the changes in proteins noted to occur during aging (8). The aging of collagen, for example, can be mimicked *in vitro* by the crosslinking induced by glucose (9). In addition, the trapping of other proteins to collagen may occur by a crosslinking reaction and account for the accumulation of albumin and antibodies in kidney basement membrane (9).

The lack of detailed information on the chemistry of the late-stage Maillard process has made it difficult to form a clear understanding of the possible roles of this reaction in structural and functional changes in tissues. For some time we have sought to identify specific fluorescent chromophores derived from the reaction of glucose with amino groups of polypeptides. In the present paper, we report the isolation and characterization of a highly fluorescent yellow-brown product from the acid hydrolysates of bovine serum albumin and polylysine that have undergone nonenzymatic browning *in vitro* with glucose. By chemical and spectroscopic analysis and independent chemical synthesis we have identified this compound as 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (FFI). The similarity of the fluorescence properties of FFI to the fluorescence of nonenzymatically glycosylated polypeptides from which it is derived strongly suggests that FFI is representative of a chromophore present in the intact polypeptides. The possible pathways for the formation of this chromophore and its potential role in the crosslinking and aging of proteins will be discussed.

MATERIALS AND METHODS

Preparation of Maillard Products. Poly(L-lysine) (1 g; Sigma; M_r 30,000–70,000) was dissolved in 50 ml of sodium phosphate buffer (pH 7.5, 0.5 M Na). D-Glucose (50 g) was added and the solution was incubated at 37°C for 28 days. The solution was dialyzed extensively against distilled water and lyophilized to yield 1.9 g of brown polylysine (B-polylysine). Bovine serum albumin (5 g; Sigma) was allowed to react under analogous conditions to yield 5.2 g of lyophilized brown bovine serum albumin (B-albumin).

Isolation of the Fluorescent Product. B-polylysine (1.5 g) was dissolved in 50 ml of 6 M HCl. The solution was deoxygenated by perfusion with nitrogen and hydrolyzed for 10 hr at 110°C. The hydrolysate was evaporated to dryness in a stream of nitrogen gas at 40°C and then redissolved in 50 ml of 0.1 M HCl. This solution was extracted five times with 50 ml of isobutyl alcohol and then five times with 20 ml of chloroform. The organic extracts were discarded. The aqueous phase was adjusted to pH 11 with ammonium hydroxide (25%) and the solution was then extracted five times with 20 ml of chloroform. The chloroform extracts were combined and evaporated in a stream of nitrogen (yield, 35 mg). This material was redissolved in 2 ml of chloroform and applied to a 10 × 200 mm column filled with silica gel (Woelm, Esch-

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Abbreviations: FFI, 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole; B-polylysine, brown polylysine; B-albumin, brown bovine serum albumin.

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wege, Federal Republic of Germany), which had been activated at 150°C overnight and pre-equilibrated with chloroform. The column was washed with 20 ml of chloroform and then the material was eluted with chloroform/methanol (1:1). The yellow fractions were pooled and evaporated to dryness with nitrogen to yield 20 mg of fluorescent compound. B-albumin (5 g) was hydrolyzed and worked up under the same conditions described for B-polylysine to yield 2.5 mg of fluorescent compound.

Analyses. The isolated compounds were analyzed by TLC on silica gel G using the solvent system ether/methylene chloride (1:4). The spots were visualized by UV light. HPLC analyses were carried out on prepacked silica gel columns (Porasil, 4 × 300 mm, Waters Associates), with heptane/isopropanol (3:2) as the mobile phase at a rate of 1 ml/min using a Hewlett Packard 1084B liquid chromatograph equipped with a variable wavelength detector. Absorption and fluorescent spectra were taken on a Hewlett Packard 8450A spectrophotometer and on a Perkin-Elmer 204 spectrofluorimeter, respectively. Absorption spectra were taken at a concentration of 1 mg/ml in 0.05 M sodium phosphate buffer (pH 7.4). Fluorescence spectra were taken at 0.1 mg/ml in the same buffer. Acid hydrolysates were analyzed by ion-exchange TLC on FIXION chromatosheets (Chromatronix, Mountain View, CA), as described (10), using furosine and pyridosine standards kindly provided by P. A. Finot.

^{1}H NMR spectra were taken in $CDCl_3$ on a Nicolet NTC-300 (wide-bore) spectrometer operated at 300 MHz in the Fourier transform mode. Chemical shifts are in ppm downfield from tetramethylsilane.

Mass Spectrometry. A DuPont 21-492 double focusing magnetic deflection mass spectrometer modified for optional chemical ionization operation, equipped with a Varian 1400 gas chromatograph and coupled to a VG 2035 data system (VG Datasystems, Altringham, U.K.), was used. For low-resolution (R = 1000) measurements, a combined electron-impact/chemical ionization ion source was used, with a source temperature of 180°C. Samples were introduced on a ceramic probe tip (‡) or via gas chromatograph (column: 1% SE-30, 1.8 m long; oven temperature: 100°C for 3 min, then programed to 250°C at 10°C/min).

For chemical ionization experiments isobutane was used as the reactant gas. Its pressure in the ion source was about 0.3 torr. For high-resolution measurements (R = 5000) a dedicated electron impact source was used and samples were introduced in glass capillary probe cups. Twenty to 25 scans from three runs were averaged for the calculation of the elemental composition. Fission fragment ionization mass spectra were obtained on the Rockefeller University custom-built time-of-flight mass spectrometer (11).

Chemical Derivatization for Mass Spectrometry. Derivatizations of FFI were carried out in 1-ml conical septum vials using 50 μg of B-polylysine-derived FFI as starting material. Methoxime formation was carried out by adding 100 μl of methoxyamine hydrochloride (Sigma) as a 10% solution in pyridine and incubating the solution at 80°C for 1 hr (12). Sodium borohydride reduction was carried out by adding 2 mg of solid $NaBH_4$ (Sigma) to 100 μl of aqueous suspension of FFI (12). After 20 min at room temperature the excess reagent was neutralized with a drop of glacial acetic acid. The solution was rendered alkaline with excess ammonium hydroxide, evaporated to dryness, and the reduced derivative was extracted from the residue with CH_2Cl_2. Monomethylation was carried out with 100 μl of dimethylformamide dimethylacetal (Eastman) at 60°C for 2 hr (12). A portion of the monomethylated derivative was evaporated to dryness and treated with 100 μl of methyl iodide at 37°C overnight to give a dimethylated derivative.

Chemical Synthesis of FFI. A solution of 2-furylglyoxal hydrate (710 mg) in a mixture of 10 ml of ethanol and 3 ml of

‡Bencsath, F. A. & Field, F. H., *Abstracts of the Twenty-Ninth Annual Conference on Mass Spectrometry and Allied Topics*, May 24–29, 1981, Minneapolis, MN, p. 587.

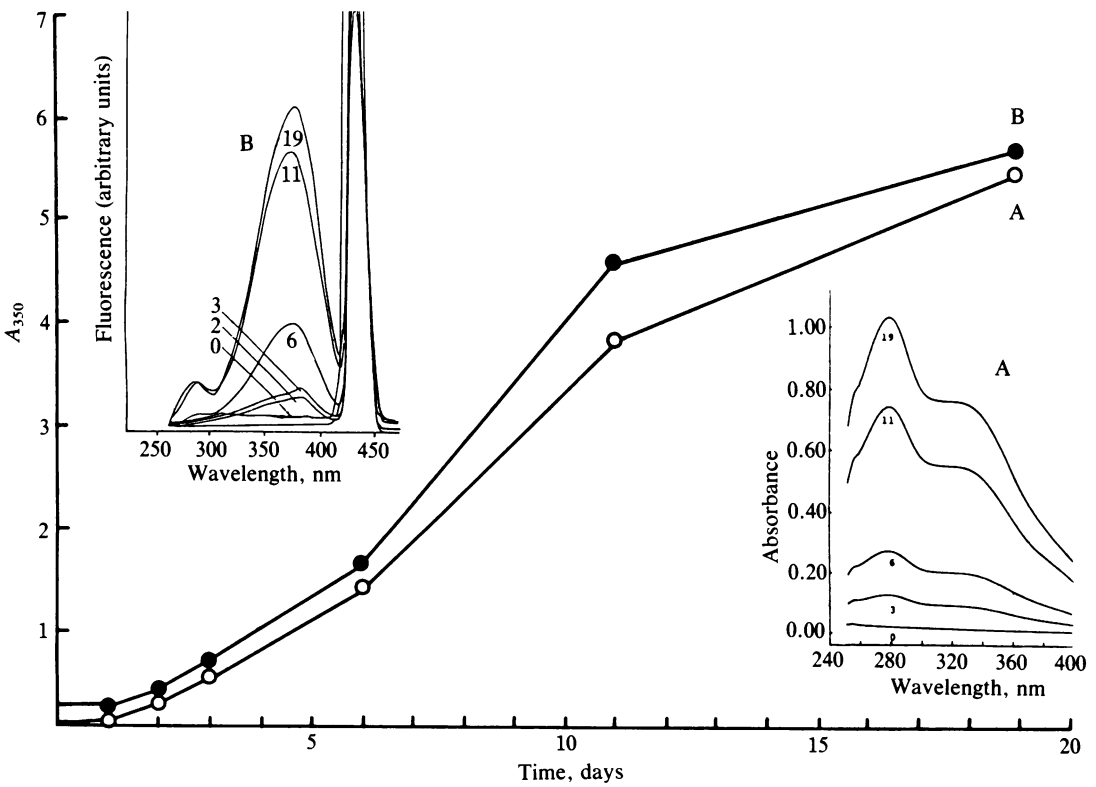

FIG. 1. Time courses of increasing UV absorbance (*A*) and increasing fluorescence (*B*) at 440 nm in the reaction of poly(L-lysine) with glucose.

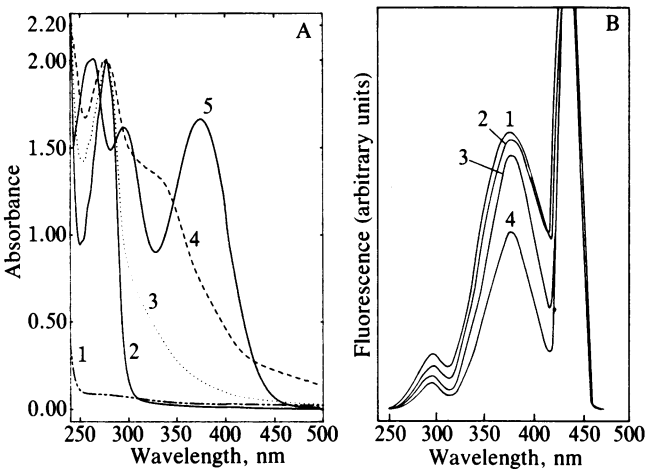

FIG. 2. Absorption and fluorescence spectra of nonenzymatically browned polypeptides and isolated FFI. (*A*) Absorption spectra: curve 1, poly(L-lysine); curve 2, bovine serum albumin; curve 3, B-albumin; curve 4, B-polylysine; curve 5, FFI isolated from B-polylysine. (*B*) Fluorescence excitation spectra recorded at 440-nm emission wavelength: curve 1, B-polylysine; curve 2, B-albumin; curve 3, FFI isolated from B-polylysine; curve 4, FFI isolated from B-albumin.

water was treated dropwise with 2 ml of concentrated aqueous NH_3. After 80 min the brown solution was partitioned between H_2O and diethyl ether/dichloromethane (1:1). The organic layer was washed twice with water, dried with Na_2SO_4, and filtered. The filtrate was concentrated and the residue was chromatographed on silica gel with ether/CH_2Cl_2 (6:94) as eluant. Fractions containing material that coeluted on TLC with FFI from glucosylation experiments were pooled and concentrated, and the residue was recrystallized from ether. Pure FFI (82 mg, 12%) was obtained as golden-yellow crystals with a brownish sheen: melting point, 176–177°C; analysis calculated for $C_{12}H_8N_2O_3$: C, 63.2%; H, 3.5%; N, 12.3%; found: C, 63.1%; H, 3.6%; N, 12.2%. The spectroscopic and chromatographic properties of this material were identical with those of the FFI samples derived from B-polylysine and from B-albumin.

RESULTS

Maillard Reaction of Polypeptides *in Vitro*. Poly(L-lysine) and bovine serum albumin were incubated in concentrated, well-buffered glucose solution at 37°C for 4 weeks, producing extensive Maillard browning. After dialysis and lyophilization, the weight gains observed in the glycosylated polypeptides were consistent with reaction of a large proportion of the free amino groups [90% increase for poly(L-lysine), 4% increase for serum albumin].

The brown poly(L-lysine) has a UV absorption maximum at 278 nm, with a prominent shoulder centered around 330–335 nm (Fig. 1). Tailing of this shoulder into the visible wavelengths is responsible for the brown color of the material. From the time course shown in Fig. 1, it may be seen that the rate of color formation increased during the first 10 days of glucose incubation and slowed thereafter, probably due to saturation of the available reaction sites.

Table 2. Main ions present in electron-impact mass spectra FFI and its chemical derivatives

Compound	Main ion (*m/z*)	Identity
FFI	228	$M^{+\cdot}$
(underivatized)	106	$(M - C_4H_4O)^{+\cdot}$ (loss of furan)
	95	$C_5H_3O_2$ (furoyl cation)
FFI + methoxyamine	257	$M^{+\cdot}$ of FFI-methoxime
FFI + $NaBH_4$	230	$M^{+\cdot}$ of (FFI + 2H)
	212	$(M^+ - H_2O)^{+\cdot}$
FFI + dimethylformamide dimethylacetal	242	$M^{+\cdot}$ of monomethyl-FFI
Monomethyl-FFI + MeI	257*	M^+ of dimethyl-FFI cation

*Obtained by fission fragment ionization mass spectrometry.

The excitation fluorescence spectrum of B-polylysine, measured at the emission maximum of 440 nm, shows a major excitation maximum at 370 nm and a minor maximum at 290 nm. These maxima do not correspond with the bands in the UV-visible absorption spectrum. This absence of correlation suggested to us that the fluorescence was primarily associated with a subclass of Maillard intermediates that might be sufficiently well defined for chemical isolation and analysis.

Isolation of a Fluorescent Chromophore from B-Polylysine and B-Albumin. Standard acid hydrolysis conditions (6 M HCl, 100–110°C) were used for cleavage of the browned polypeptides. Hydrolysis of B-polylysine yielded predominantly lysine, furosine, and pyridosine based on ion-exchange TLC comparison with authentic materials; these are known to be the major breakdown products of ε-deoxyfructosyl-lysine under acid hydrolytic conditions. Although fluorescence could be detected in the crude hydrolysates from the browned polypeptides, there appeared to be substantial quenching by UV-absorbing by-products.

Chromatography on arenesulfonic acid-type cation-exchange resin proved to be unsuitable for separation of the fluorescent material; its failure to elute cleanly from this resin suggested that it was hydrophobic and/or weakly basic. After mild alkalinization of the aqueous hydrolysates with ammonia or triethylamine, much of the fluorescence could be extracted into chloroform, while most of the brown color remained in the aqueous phase. By column chromatography of the chloroform extract on silica gel, it was possible to purify a single major fluorescent compound; this material had a R_f of 0.50 on silica gel TLC using ether/dichloromethane (1:4) as solvent.

The fluorescence excitation spectra of B-polylysine and B-albumin and of the purified chromophore derived from B-polylysine and B-albumin, respectively, are compared in Fig. 2*B*. The qualitative identity of these spectra is evident. The absorbances in the UV spectrum of the isolated chromophore (Fig. 2*A*, curve 5) clearly correlate well with the two fluorescence excitation maxima. These data suggest that the isolated material incorporates a principal Maillard-derived chromophoric center that has survived hydrolytic cleavage from the browned polypeptides without significant loss of chemical integrity.

Table 1. High-resolution electron-impact mass spectrometry of FFI

Observed mass, *m/z*	*n*	Computed mass, *m/z*	Δ*m* (millimass units)	Composition	Identity
228.0521 ± 0.0020	25	228.0534	1.4	$C_{12}H_8N_3O_2$	Molecular ion ($M^{+\cdot}$)
160.0223 ± 0.0022	23	160.0272	5.0	$C_8H_4N_2O_2$	$M^{+\cdot}$ − furan
95.0140 ± 0.0018	25	95.0133	−0.7	$C_5H_3O_3$	Furoyl ion

Observed data are given as mean ± SEM (*n* = number of determinations).

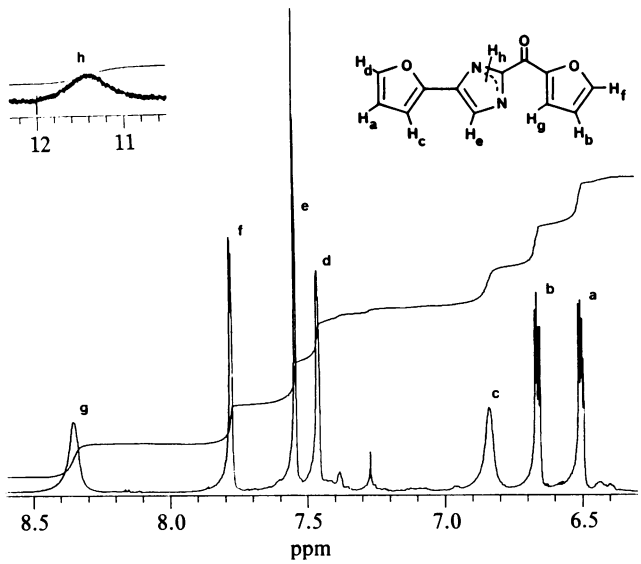

FIG. 3. ^{1}H NMR spectrum of FFI isolated from B-polylysine ($CDCl_3$, 300 MHZ).

The more readily available B-polylysine-derived material was principally used for the structure determination studies that follow; subsequent analysis of the B-albumin-derived chromophore under like conditions served to prove its identity with B-polylysine-derived and chemically synthesized material (see below).

Mass Spectroscopic Analysis of the Isolated Chromophore. The electron-impact mass spectrum of the chromophore isolate showed a relatively simple pattern dominated by three abundant ions m/z 228 (100%), 181 (33%), and 95 (88%). The isobutane chemical ionization mass spectrum consisted essentially of a single ion at m/z 229, corresponding to a M_r of 228 for the parent species. The elemental composition of the major ions was determined by averaged high-resolution electron-impact mass spectroscopy (Table 1). The molecular ion was found to have the formula $C_{12}H_8N_2O_3$. The formula of the m/z 95 fragment is that of the furoyl cation. The other major fragment, m/z 160, corresponds to the loss of the elements of neutral furan from the parent ion. This fragmentation pattern is consistent with a structure in which a 2-furanyl group and a group of formula $C_7H_5N_2O$ are linked together through a carbonyl group.

Chemical Derivatization of the Isolated Chromophore. The chromophore material was found to form a monomethoxime derivative on reaction with methoxyamine and underwent reduction with sodium borohydride with uptake of two hydrogens to yield a colorless derivative, consistent with the presence of a conjugated, noncarboxylic carbonyl group. Methylation studies demonstrated the presence of two nucleophilic sites that are differentially susceptible to alkylation. The dimethyl derivative had chromatographic and mass spectrometric properties of a quaternary methiodide salt (Table 2).

^{1}H NMR Spectrum of the Isolated Chromophore. Fig. 3 shows the ^{1}H NMR spectrum of the chromophore material from B-polylysine. Double irradiation experiments demonstrated that resonances a, c, and d comprise a group of three mutually coupled protons and that resonances b, f, and g comprise another similar but distinct group. Although one resonance in each group was anomalously broadened, there was sufficient structure in the other resonances to allow the estimation of the three coupling constants for each group. These constants fall well within the normal range observed for coupling constants in 2-substituted furan rings (Table 3). Thus, it appeared that, in addition to the furoyl group indicated by the mass spectral data, another chemically distinct 2-substituted furan ring was present. Subtracting these from the molecular formula leaves the elements of disubstituted imidazole. The presence of an imidazole ring would account for the presence of two differentially nucleophilic centers. The tautomerism of the NH proton between two nonequivalent imidazole nitrogens, with a rate similar to the proton relaxation times, would explain the broadening of the 3-position hydrogen resonances in the furan rings. These factors together with mechanistic considerations for the formation of the chromophore lead to the assignment of the structure as 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole, the two tautomeric structures of which are shown in Fig. 4. The fact that the signal for H_e on the imidazole ring is not broadened by the tautomerism may be due to fortuitous similarity of its chemical shifts in the two tautomers or to effects on its relaxation time by the adjacent nitrogen quadrupole.

Table 3. H-H coupling constants of FFI furan rings

Coupling type	Observed coupling, Hz		
	Furanyl ring	Furoyl ring	Typical range, Hz*
Furan H2–H3	J_{ac} = 3.4	J_{bg} = 3.6	3.1–3.8
Furan H3–H4	J_{ad} = 1.8	J_{bf} = 1.6	1.3–2.0
Furan H2–H4	J_{cd} = 0.7	J_{fg} = 0.5	0–1

*From ref. 13.

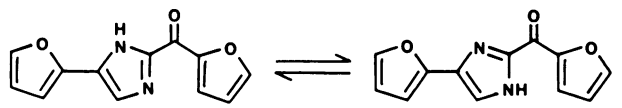

FIG. 4. Tautomers of FFI.

Identity of the Isolated Chromophore with Synthetic FFI. As a final confirmation of the assigned structure, we prepared FFI by an independent chemical method. A search of the literature failed to reveal any previous report on FFI. However, a number of diphenyl analogs such as 2-benzoyl-4(5)-phenyl-1*H*-imidazole have been described (14); these are prepared by the reaction of phenylglyoxal derivatives with ammonia. The analogous reaction of furylglyoxal with

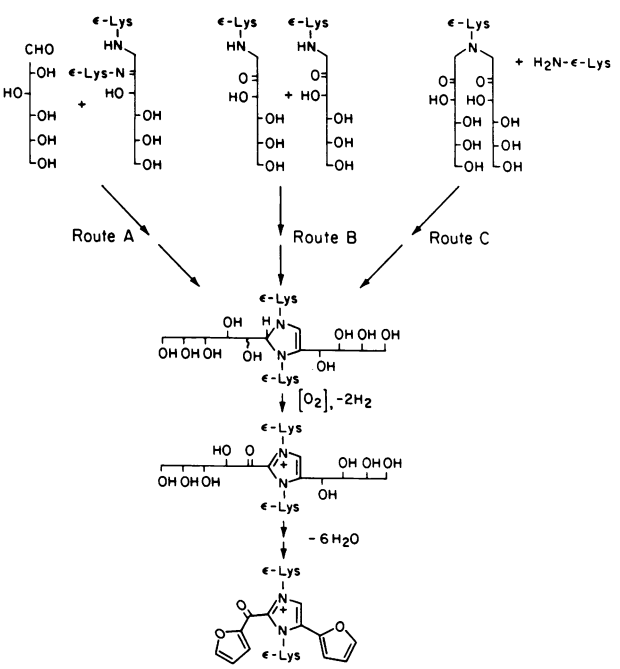

FIG. 5. Possible routes for the formation of FFI-like protein crosslinks from initial Amadori adducts of glucose and ε-lysyl residues.

ammonia in aqueous ethanol provided a modest yield of FFI, identical in all respects with the glycosylation-derived materials.

DISCUSSION

The incubation of proteins with glucose leads to a number of physicochemical changes, including the formation of brown color, fluorescence, and crosslinking. In the present communication, we report the isolation and characterization of a yellow-brown fluorescent chromophore, FFI, which appears to represent a cleaved form of a key intermediate in these processes. This compound is a condensation product of two glucose molecules and two lysine-derived amino groups into a conjugated system of three aromatic heterocycles. The fluorescence properties of FFI are remarkably similar to those of the nondialyzable pigments bound to albumin and to polylysine that have undergone extensive nonenzymatic browning with glucose. This similarity implies that there is a close analogy between the structure of FFI and that of the chromophoric centers present in the fluorescent pigments. During subsequent acid hydrolysis, ε-lysyl residues could cleave from the imidazole nitrogens via internal nucleophilic attack of their released α-amino groups at the ε-positions, forming FFI-like materials and pipecolic acid.

There are a number of different sequences in which two glucose molecules and two lysine ε-amino groups could interact to form a tetrasubstituted imidazole system. Three of these possibilities are illustrated in Fig. 5, (routes A–C). The possibility of route C is suggested by our previous observation of a product of the incubation of glucose with valine, which had two attached sugar residues (4). Both routes A and C, in which fructosyllysine species interact with another amino group, might explain the attachment of proteins to other proteins that have previously undergone nonenzymatic browning (9).

Cyclization to a tetrasubstituted dihydroimidazole (i.e., imidazoline) structure and its subsequent autoxidation to a quaternary imidazolium species would appear to be crucial steps in the formation of FFI. The dihydroimidazole intermediate would be a highly electron-rich center, and its autoxidation under conditions of physiological oxygen tension in low-turnover proteins is not difficult to envision. At present it is difficult to assess the extent or rate at which subsequent dehydrations would occur *in vivo* to form one or both of the furan rings observed in FFI; such dehydrations could be facilitated in instances in which the crosslinking of proteins via imidazole formation results in the creation of a hydrophobic microenvironment around the glucose-derived polyhydroxylic side chains.

The identification of FFI as a specific product that is characteristic of the late-stage Maillard reaction provides a starting point from which further systematic studies of chemistry of this process can proceed. Also, the measurement of a specific compound, FFI, derived from specific Maillard intermediates, may provide a new approach to quantitating the primary sites of crosslinking formed during aging of proteins and nucleic acids (8, 15). In the clinical study of aging and of diabetes, measurement of FFI may allow assessment of the time-averaged exposure of long-lived structural tissues to glucose over extended periods (months to years), analogous to the current utility of hemoglobin A_{1c} levels in estimating glucose exposure over 3- to 4-week periods. In addition, the quantitation of FFI formed *in vivo* in conjunction with concurrent clinical observations should provide new insight into specific roles of Maillard products in the complications of aging and diabetes.

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1. Maillard, L. C. (1912) *C. R. Acad. Sci. (Paris)* **154,** 66–68.
2. Finot, P. A. (1982) in *Modification of Proteins*, eds. Feeney, R. E. & Whitaker, J. R. (American Chemical Society, Washington, DC), Vol. 198, pp. 91–124.
3. Bunn, H. F., Haney, D. N., Gabbay, K. H. & Gallop, P. H. (1975) *Biochem. Biophys. Res. Commun.* **67,** 103–109.
4. Koenig, R. J., Blobstein, S. H. & Cerami, A. (1977) *J. Biol. Chem.* **252,** 2992–2997.
5. Monnier, V. M. & Cerami, A. (1983) in *Maillard Reaction in Food and Nutrition*, ed. Waller, G. A. (American Chemical Society, Washington, DC), Vol. 215, pp. 431–448.
6. Monnier, V. M. & Cerami, A. (1983) *Biochim. Biophys. Acta* **760,** 97–103.
7. Monnier, V. M., Kohn, R. R. & Cerami, A. (1983) *Proc. Natl. Acad. Sci. USA* **81,** 583–587.
8. Monnier, V. M. & Cerami, A. (1981) *Science* **211,** 491–493.
9. Brownlee, M., Pongor, S. & Cerami, A. (1983) *J. Exp. Med.* **158,** 1739–1744.
10. Pongor, S., Kramer, J. & Ungar, E. (1980) *J. High Resolut. Chromatogr. Chromatogr. Commun.* **3,** 93–94.
11. Chait, B. T., Agosta, W. C. & Field, F. H. (1981) *Int. J. Mass Spectrom. Ion Phys.* **39,** 339–366.
12. Blau, K. & King, G. S. (1977) *Handbook of Derivatives for Chromatography* (Heyden, London).
13. Silverstein, R. M. & Bassler, G. C. (1967) *Spectrometric Identification of Organic Compounds* (Wiley, New York), 2nd Ed., p. 145.
14. Schubert, W., Hellwig, A. & Bleichert, J. L. (1964) *J. Prakt. Chem.* **24,** 125–131.
15. Bucala, R., Model, P. & Cerami, A. (1983) *Proc. Natl. Acad. Sci. USA* **81,** 105–109.