N-(2-Furacyl)glycine—A Furan Amino Acid Derived from Fructose-Glycine

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A furan amino acid, eluted between alanine and cystine during ion exchange chromatography, was isolated from a hydrolyzate (6*N* HCl) of fructose-glycine. Formed in a yield approximating 0.2 mole for each mole of free glycine released, this unexpected side-product was identified as *N*-(2-furacyl)-glycine. It was obtained as crystalline free amino acid ($C_8H_9NO_4$) and hydrochloride salt. Structural assignment was based on microanalysis, spectral studies, and chemical degradation; the structure was confirmed by synthesis from furacyl bromide and ethyl glycinate. Although very resistant to acid cleavage, this furan derivative of glycine was quantitatively cleaved to free glycine in bicarbonate solution at 24° C. Since fructose-amino acids are known to occur in foods, the commonly employed hydrolytic procedures with 6*N* HCl may produce acid-resistant *N*-furacyl-amino acids in acid-hydrolyzed foods.

During a study of the reduction of fructose-amino acids by sodium borohydride (Lipton *et al.*, 1970), fructose-amino acids and their corresponding reduction products (sugar alcohol-amino acids) were subjected to hydrolysis with 6*N* HCl. The reduction products were found to be relatively stable to acid, as compared with the fructose-amino acids. Fructose-glycine (FG) was completely converted into a mixture of glycine and a second previously unidentified amino acid. The latter was isolated as a crystalline free amino acid ($C_8H_9NO_4$) and hydrochloride salt, and identified as *N*-(2-furacyl)glycine (I). The isolation, identification, and synthesis of (I) are detailed in this report. Its relation to studies of nonenzymic browning of foods in model systems (Hodge, 1953) is discussed.

EXPERIMENTAL

Materials and Methods. Fructose-glycine (FG) was prepared as previously described (Hagan *et al.*, 1970). Furyl methyl ketone, 2-furoic acid, and ethyl glycinate hydrochloride were commercial products. Furacyl bromide was prepared (Brown, 1937) by reacting furyl methyl ketone with bromine in carbon disulfide solution. Free glycine ethyl ester was prepared from the corresponding hydrochloride by extracting with ether in the presence of excess aqueous NH_4OH.

A Phoenix Biolyzer, Model 3000, was used for quantitative amino acid analyses. The acidic and neutral amino acids were separated on a 60 cm × 0.9 cm column of cation exchange resin, using 0.2 *M* sodium citrate buffers (Spackman *et al.*, 1958) and a change from pH 3.26 to pH 4.26 buffer at an elution volume of about 120 ml. An Infotronics, Model CRS-10, electronic integrator was used for automatic computation of peak times and areas. (The various mono-*N*-substituted amino acids such as fructose-amino acids, sugar alcohol-amino acids, and *N*-furacyl-amino acids gave a normal ninhydrin reaction, with ratios of the 550 nm to 440 nm absorbance close to the values for the corresponding free amino acids.)

Paper chromatography of amino acids and their derivatives was on Whatman No. 1 filter paper, using an ascending technique. Solvent systems used were either butanol:acetic acid:water (12:3:5) or 2-butanone:propionic acid:water (15:5:6), stated on a volume ratio basis. Amino acids and derivatives were visualized by spraying with 0.1% ninhydrin in acetone, followed by exposure to a stream of hot air from a heat gun. Fructose-amino acids and *N*-furacyl-amino acids appeared as red zones, when sprayed first with 1% triphenyl tetrazolium chloride (TCC) in ethanol, and then with 0.5% sodium hydroxide in ethanol. *N*-furacyl-amino acids were also located as ultraviolet-quenching zones when the unsprayed dried chromatogram was viewed in the dark with a Mineralite shortwave ultraviolet lamp. (*N*-Furacyl-amino acids became visible on the unsprayed dried chromatograms as yellowish to light tan zones after storage for several days at room temperature.)

Ultraviolet spectra were obtained on a Cary Model 14 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer Model 521 spectrophotometer in pressed disks of KBr at a sample concentration of approximately 0.3%. Mass spectra were obtained on an Hitachi RMU-6E medium resolution spectrometer at an ionizing voltage of 70 e.V. (or 25 e.V.) with an ion source temperature of 230° C. The sample was introduced by use of the direct probe at a block temperature of 150° C. Nuclear magnetic resonance (nmr) spectra were obtained on a Varian Model A-60 spectrometer at 60 MHz. Data are presented as chemical shifts (σ values), whose values are approximated by the use of center-of-gravity estimates in the case of higher order multiplets. Samples were titrated for determination of neutral equivalent and pK values, using a Radiometer SBR-2C Titrigraph and allied equipment. Melting points were determined on a Fisher-Johns hot stage, and are reported uncorrected.

Isolation of *N*-(2-furacyl)glycine (I). FROM FG BY ION-EXCHANGE CHROMATOGRAPHY. Fructose-glycine (2.5 g, 10.5 mmoles) was dissolved in 100 ml of 6*N* HCl and boiled for 5 hr under a reflux condenser. The hydrolyzate was decolorized (Darco G 60) and concentrated *in vacuo* to a faintly yellowish solid residue. This was dissolved in 100 ml of water and adsorbed (flow rate 2 ml per min) on a bed (3.5 cm deep, 4.3 cm diameter) of cation exchange resin (Dowex 50-X8, H^+ form, 100 to 200-mesh). The resin was then washed with 140 ml of water and eluted with trichloracetic acid (TCA) (350 ml of 0.25 *M* followed by 1200 ml of 0.5 *M*, collection in 10 ml fractions). Free glycine emerged first (approximate elution volume of 0.5 *M* TCA was 60–280 ml) followed by I (approximate elution volume of 0.5 *M* TCA was 600–1000 ml). The fractions containing I were freed of TCA by extraction with ether (five times, 0.2 volume each) and evaporated *in vacuo*. Addition of 3 volumes of ethanol to the syrupy concentrate afforded colorless crystalline I: Wt.

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200 mg; m.p. (after recrystallization from ethanol-water) *ca.* 210° C dec.; $\lambda_{max}^{H_2O}$ (pH 1.0) 282 nm (ε 15,600) and 229 nm (ε 2240), min 244 nm (ε 1550); ν_{max}^{KBr} 2900–3200m, 1671s, 1620s, 1569m, 1463m, 1393m, 1340s, 1287m, 1084m, 1016m, 1001m, 938m, 912m, 898m, 880m, 767m cm^{-1}; mass spectrum: *m/e* 183 (2.7), 165 (3.5), 110 (90), 95 (19), 88 (100), 81 (15), 68 (4), 60 (25), 42 (100); observed pK_b, 7.55, *vs.* 9.6 for glycine and 8.1 for FG. *Anal.* Calcd. for $C_5H_9NO_4$: C, 52.46; H, 4.95; N, 7.65; neut. equiv. 183.2. Found: 52.81; H, 5.40; N, 8.01; neut. equiv. 181.6. (C, H, and N analyses of this sample only were by Aldridge Associates, Washington, D.C.)

DIRECT CRYSTALLIZATION OF I·HCl FROM HYDROLYZATE OF CRUDE CATIONIC FRACTION OF HEATED GLUCOSE-GLYCINE MIXTURE. Glycine (75 g, 1 mole) was added with stirring to a syrup of glucose (454 g of monohydrate, 2.3 moles dissolved in 100 ml of hot water). After heating for 20 min at an internal temperature of 95° C, the now almost black mixture was cooled in ice, diluted to 8 l. with water, and directly adsorbed (rate 75 ml per min) on a bed (10 cm deep, 16 cm diameter) of cation exchange resin (Dowex 50W-X8, H^+ form, 100 to 200-mesh). Noncationic substances were removed by washing the resin with an additional 8 l. of water. Amino acids were eluted with aqueous ammonia (1.8*N*) at a rate of 50 ml per min, and occurred between 2.5 and 4.5 l. The dark syrup obtained by concentrating these fractions *in vacuo* was diluted with water to a volume of 250 ml, mixed with an equal volume of concentrated HCl, and heated 1.5 hr under a reflux condenser. After decolorizing with 25 g of Darco G 60, the hydrolyzate was evaporated *in vacuo* to a brown semi-solid residue, which had a very strong maple syrup-like odor. This residue was dissolved in 100 ml of hot water, and upon cooling it afforded two crops of crude I·HCl (26.3 g). Examined on the amino acid analyzer at this stage, the product was a mixture of approximately 4 parts of glycine and 1 part of I. By recrystallizing several times from water (strongly acidified with HCl), colorless needles of pure I·HCl were obtained: m.p. > 180° C dec.; ν_{max}^{KBr} 2700–3300(br)s, 2623s, 2402m, 1733s, 1660s, 1569s, 1462s, 1390s, 1300s, 1220s, 1081s, 1030s, 990s, 930m, 918m, 893s, 880m, 842s, 769s, 588m, 507m, cm^{-1}; nmr spectrum (δ) from external tetramethylsilane [trifluoroacetic acid-d (TFA-d)]: 4.03 (2H,s,N-CH_2COO), 4.50 (2H,s,Furacyl-CH_2N), 6.32 (1H,m,Furan-C_4H), 7.12 (1H,m,Furan-C_3H) and 7.37 (1H,m,Furan-C_5H). $J_{3,4}$ = 4.0 Hz; $J_{4,5}$ = 1.6 Hz; $J_{3,5}$ < 1.0 Hz; mass spectrum: 184 (2.9) M + 1, 183 (2.4) M^+, 165 (3.5), 110 (60), 95 (20), 88 (100), 81 (13), 68 (4.4), 60 (29), 44 (11), 42 (73), 38 (3.5) and 36 (10). (M^+ designates the molecular ion of free I. Apparently the hydrochloride readily dissociates to free I.) *Anal.* Calcd. for $C_5H_{10}NO_4Cl$: C, 43.75; H, 4.59; N, 6.38; Cl, 16.19. Found: C, 43.76; H, 4.45; N. 6.28; Cl, 15.99.

Synthesis of *N*-(2-furacyl)glycine·HCl. Furacyl bromide (1.8 g, 0.0096 mole), dissolved in 10 ml of absolute ether, was slowly added to an ice-cooled solution of ethyl glycinate (2.0 g, 0.019 mole) in 40 ml of absolute ether. The stirred mixture was kept in ice for 30 min, during which a colorless solid gradually precipitated. After standing for 30 min at room temperature, the mixture was filtered to remove the precipitate of ethyl glycinate hydrobromide. By evaporating the filtrate *in vacuo*, crude furacylglycine ethyl ester was obtained as a red solid residue. (Due to the potent lachrymatory property, a laboratory hood should be used.) This crude ester was directly hydrolyzed by boiling 30 min in 30 ml of 6*N* HCl. The hydrolyzate was decolorized with 1 g of Darco G 60, filtered, and concentrated *in vacuo* to a volume of 15 ml. The crystals which separated upon cooling in ice were filtered, washed with 3*N* HCl, and dried (wt. 1.16 g, 55% yield based on the furacyl bromide). Analysis on the amino acid analyzer showed the composition to be 98% of I and 2% of glycine. By recrystallizing from 3*N* HCl, glistening colorless crystals of I·HCl were obtained: m.p. > 180 dec. *Anal.* Calcd. for $C_5H_{10}NO_4Cl$: C, 43.75; H, 4.59; N, 6,38; Cl, 16.19. Found: C, 43.67; H, 4.48; N, 6.67; Cl, 15.71. (The infrared and nmr spectra were essentially identical to those of isolated I·HCl.)

***N*-Acetyl Furacylglycine (II).** A mixture of 100 mg of I·HCl and 50 mg of sodium bicarbonate dissolved in 1 ml of water was heated 30 min in a large excess of acetic anhydride. After evaporation *in vacuo* the syrupy residue was taken up in ethanol, sodium chloride was filtered off, and upon reevaporation the oily water-insoluble residue was dissolved in methanol. Examination on the amino acid analyzer indicated the absence of both free I and glycine. By hydrolyzing a small sample of this oil in 6*N* HCl, a peak corresponding to I reappeared in the amino acid analysis. Since efforts to crystallize II were unsuccessful, the solvent was removed *in vacuo* and the mass spectrum of the crude II was obtained: *m/e* 225 (2.5) (M^+), 207 (29), 183 (18), 165 (41), 137 (12), 130 (9) (M-95), 95 (20), and 88 (100).

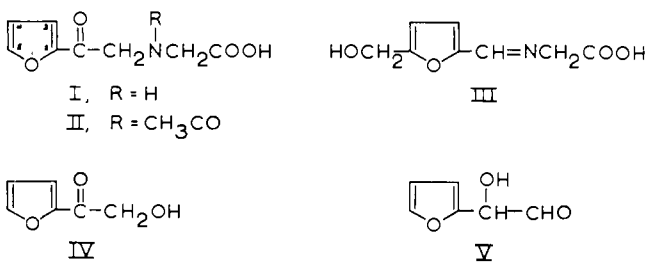
Oxidation of I to 2-Furoic Acid. An aqueous solution of I was boiled for 3 min with alkaline permanganate, then freed of precipitated MnO_2 and excess permanganate, acidified, and extracted several times with ether. After decolorizing with carbon, the dried ether was evaporated, affording colorless needle rosettes, identical with authentic 2-furoic acid. Paper chromatography: R_f 0.84 in butanol:acetic acid:water system *vs.* R_f 0.39 for I, detected as ultraviolet quenching zones, unresolved from authentic 2-furoic acid in this as well as in 2-butanone:propionic acid:water system (R_f 0.89).

RESULTS

Amino Acid Products of Hydrolysis of FG by 6*N* HCl. Only 2% of the initial FG survived boiling 6*N* HCl for 2 hr (Figures 1 and 2). Free glycine, which was the major product as expected, was eluted as *ca.* 84 min and comprised about 82% of the total amino acids. The previously unidentified post-glycine peak (I) was eluted at *ca.* 98 min and at 2 hr it accounted for 16% of the total. Only traces of other amino acids were visible on the chromatograms, which were limited to neutral and acidic groups (Figure 1). When hydrolyzed 18 hr in 6*N* HCl, the distribution was 88% glycine and 12% I. Paper chromatography of hydrolyzates confirmed the presence of I in addition to glycine. In the butanol-acetic acid-water system, I had R_f 0.37 *vs.* R_f 0.11 for FG, and 0.20 for glycine. In the 2-butanone-propionic acid-water system, I had R_f 0.33 *vs.* R_f 0.07 for FG and 0.12 for glycine. With the ninhydrin spray, I appeared as a peach-colored zone, which contrasted with the lavender appearance of FG and glycine.

Identification of I. Chromatography of an hydrolyzate of FG on a strong acid cation exchange resin with elution by TCA was used for the initial isolation of I in substantially pure form. Subsequently, we observed that I·HCl crystallized preferentially from the HCl hydrolyzate, and could thus be obtained pure in gram quantities, without the laborious chromatographic procedure. When isolated I was added to a standard mixture of common protein amino acids, it was eluted in the autoanalyzer at a position between alanine and cystine. We quickly suspected I to be an acid-

catalyzed dehydration product of FG from a few initial observations: the neutral equivalent of 182 (correct value is 183) suggested loss of 3 moles of water (54) from FG (MW 237); the ultraviolet absorption spectrum indicated a furan structure and upon mild alkaline hydrolysis of I, free glycine was released. Although aware of the suggestion by Gottschalk (1952) that fructose-phenylalanine was converted by acid hydrolysis to a Schiff base of 5-hydroxymethylfurfural (HMF) and phenylalanine, at the time of our isolation of I, we were unaware of the reports (Finot *et al.*, 1968; Heyns *et al.*, 1968) on the structure of N^{ϵ}-furoylmethyl-L-lysine, which was given the trivial name furosine (Finot *et al.*, 1968). Richards (1956) had postulated a corresponding Schiff base of HMF and glycine (structure III), without actually isolating or demonstrating its existence, and we first considered that possibility.



Infrared and Nmr Spectra. Structure III was excluded by infrared and nmr spectra. The infrared bands at 1671, 1569, 1393, 1016, 938, 880, and 767 in the spectrum of free I and the closely related infrared bands in the spectrum of I·HCl were consistent with the furacyl structure of I. There was no confirmation of the OH of structure III. The nmr spectrum was particularly compelling in its evidence for structure I. All protons were definitely assignable (the NH- and COOH, exchanged in the TFA-d solvent). The low field multiplets were consistent with ring protons of a 2-substituted furan, and had δ values similar to those reported for furosine (Heyns *et al.*, 1968). The methylene singlets at δ 4.03 and δ 4.50 were assigned, respectively, to the glycine methylene and the furacyl methylene. Neither of these peaks were present in the spectrum of furosine which was observed in D_2O (Finot *et al.*, 1968). This was due to a rapid exchange of the furacyl methylene protons in the D_2O. Our assignment of methylene protons was based on a comparison with the nmr spectrum of *N*-furacyl-L-methionine, also run in TFA-d (Lipton *et al.*, 1970). The presence of an identical δ 4.50 peak due to the furacyl methylene, but the absence of the δ 4.03 singlet in the furacylmethionine spectrum, allowed this distinction. The lack of exchange of hydrogens of the furacyl methylene in the strong acid TFA-d is consistent with the stability of I toward acid hydrolysis.

Mass Spectral Evidence. Low resolution spectra were obtained on both free I and on I·HCl, with similar patterns, and also a spectrum of the crude *N*-acetylfuracylglycine (II) was obtained. In all spectra, *m/e* 183 peaks were present, and in the spectrum of II the peak at *m/e* 225 (M^+) was present. As shown in Figure 3, the loss of furoyl radical resulted in ions of *m/e* 88 or 130, respectively, from I or II. The sizable *m/e* 95 furoyl ion indicated that charge resides on this fragment, even if less frequently than on the nitrogen-containing fragment pictured in Figure 3. The abundant peak at *m/e* 110 was assignable to the enolic form of furyl methyl ketone, the result of a McLafferty rearrangement.

Hydrolysis and Stability of I. The extreme susceptibility of I to alkali as contrasted with its great stability to acid soon

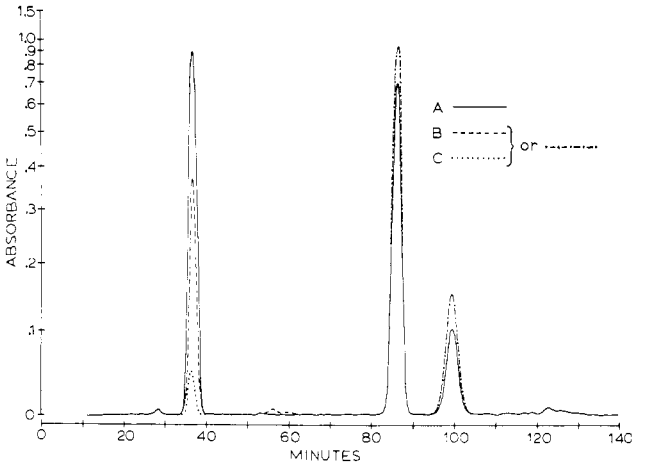


Figure 1. Chromatograms from amino acid analyzer of fructose-glycine (FG) following hydrolysis in 6*N* HCl. Hydrolysis was by boiling a solution of 2.5 g of FG in 100 ml of 6*N* HCl under reflux. Samples of 10 μl volume, applied to the analyzer, contained the equivalent of approximately 0.25 mg of FG. Times of hydrolysis: Curve A, 30 min; Curve B, 1 hr; Curve C, 2 hr. Identity of components: 29-min, trace unknown; 36-min, FG; 84-min, glycine; 98-min, furan amino acid identified here as *N*-furacylglycine (I); 122-min, trace unknown

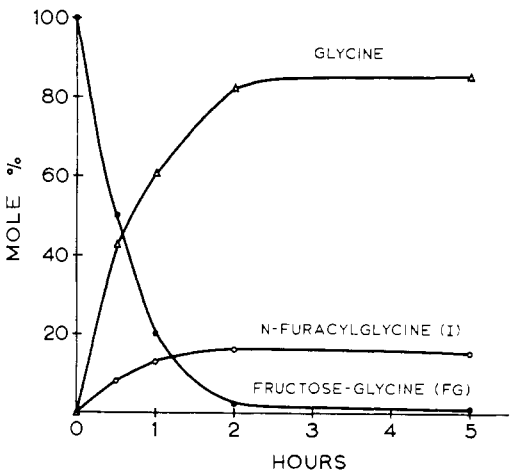


Figure 2. Composition of 6*N* HCl hydrolyzate of fructose-glycine (FG). Individual points on curves were obtained from analyses on the amino acid analyzer (see Experimental Section). Percentage of the total integrated area due to amino acid peaks (see Figure 1) in acidic and neutral regions of the chromatogram were calculated assuming the peak area for all components was identical to the peak area of glycine on a molar basis

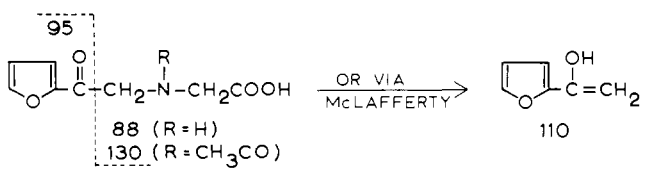


Figure 3. Mass spectral fragmentation pattern

became obvious. Merely heating free I in water for recrystallizing led to its destruction, a fact we observed soon after its isolation. As shown in Figure 4, I was quickly hydrolyzed in boiling bicarbonate solution. In bicarbonate at 24° C, I was completely hydrolyzed to free glycine upon overnight standing. On the other hand, after 18 hr in 6*N* HCl at 110° C, only about 20% of I was converted to free glycine. Under the same conditions for 70 hr, about 56%

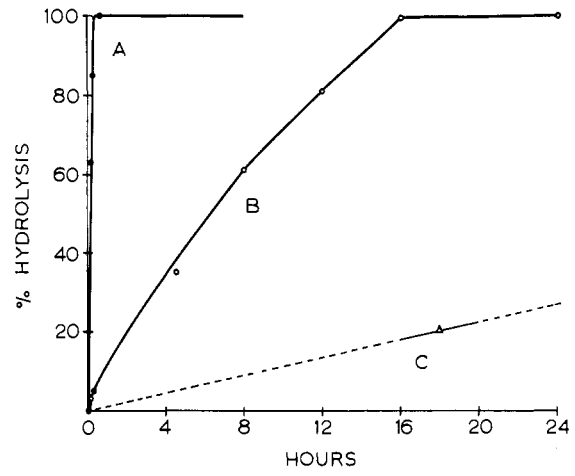


Figure 4. Hydrolysis of furacylglycine (I). Individual points on the curves were obtained from analyses on the amino acid analyzer. Percent hydrolysis was calculated from the mole% of total amino acids represented by the glycine peak, using the assumption stated in the legend to Figure 2. A solution of I containing 6.4 μmole per ml was used for the hydrolysis study. Curve A, heated in $NaHCO_3$ (pH 8.2) at 100° C; Curve B, stored at 24° C in $NaHCO_3$ (pH 8.2); Curve C, heated in 6*N* HCl solution at 110° C in sealed tube

of I was converted to free gylcine. During 7.5 months storage in 0.1*N* HCl at 4° C, less than 1% of free glycine was obtained. (FG, as was shown in Figure 2, was 98% destroyed by 6*N* HCl in 2 hr at 100° C. Also in contrast with I, after 48 hr in bicarbonate at 24° C, only 2% of the initial FG was cleaved to free glycine.)

DISCUSSION

There have been numerous studies of the Maillard reaction (Ellis, 1959; Spark, 1969) prompted by an intense interest in both nutritional and flavor aspects of the nonenzymic browning of foods (Reynolds, 1969). Nevertheless, relatively few nitrogen-containing chemical intermediates have been identified which precede degradation of the amino acid itself. The fructose-amino acids (Hodge, 1953) which are produced from the initial *N*-glycosylamines *via* the Amadori rearrangement (Hodge and Fisher, 1963) are well established intermediates. Furacylglycine (I), described in this report, furosine, and other furacyl-amino acids appear to be significant intermediates in nonenzymic browning. Furosine was first discovered as a post-arginine amino acid in acid-hydrolyzed dried skim milk (Erbersdobler and Zucker, 1966) and was suggested to be an indicator of the extent of heat damage to proteins. The ordinary hydrolytic procedures with 6*N* HCl would be expected to produce furacyl-amino acids from fructose-amino acids as previously suggested (Heyns *et al.*, 1968). Although the furfurals have long been recognized to participate in browning reactions (Stadtman, 1948), the precise role of these furans has not been defined. Various furans have been identified as end products of nonenzymic browning (Ferretti *et al.*, 1970). [Furans which were identified include flavor constituents of roasted cocoa (Van Der Wal *et al.*, 1968) and roasted coffee (Stoffelsma and Pypker, 1968).] Of the numerous isolated nonprotein amino acids which have been reviewed (Thompson *et al.*, 1969), none possessed a furan structure. Synthetic furan amino acids have been prepared (Dunlop and Peters, 1953) and include DL-β-(2-furyl)-α-alanine (Watanabe *et al.*, 1965).

5-Hydroxymethylfurfural has long been recognized (Düll, 1895) as a dehydration product of common hexoses (Newth, 1951), with evidence for its direct derivation from fructose (Haworth and Jones, 1944). An impurity detected in isolated HMF preparations was identified as 2-hydroxyacetylfuran (IV) (Miller and Cantor, 1952). The conversion of HMF into IV through acid treatment was subsequently observed (Aso and Sugisawa, 1954). The furan amino acid (I) has a formal structural relationship with IV, *i.e.*, loss of water between IV and glycine would yield I. However, when I was split in bicarbonate solution, we did not obtain IV in the form of the 2,4-dinitrophenylhydrazone derivative (Anet, 1965). A cleavage of I by reversal of the Amadori rearrangement conceivably would yield 2-(2-furyl)-glycolaldehyde (V), just as FG can be conceived to yield glucose, although in fact glucose has not been obtained. (The formation of brown-colored products, which accompanies the release of glycine from I, may proceed as rapidly as the furan fragment is released.)

I does not appear to be on the main pathway of glycine release from FG, nor does it seem likely that the principal route to I could be through recombination of free glycine and a furan derived from cleavage of FG. Only a slight trace of I was observed when equimolor parts of free glucose and glycine were refluxed in 6*N* HCl. Heyns *et al.* (1968) suggested that acid-catalyzed dehydration of fructose-amino acids to furacyl derivatives paralleled the formation of IV from fructose (Shaw *et al.*, 1967). The latter authors pointed out that IV arises from fructose *via* the 2,3-enediol and elimination of the 4-hydroxyl group. [We have observed that synthetic xylulose-glycine (Lipton *et al.*, 1970) did not yield I, or other acid-stable amino acid derivatives, a finding which is consistent with a direct dehydration route to I.]

In spite of many earlier studies of glycine-glucose browning products, I has not previously been isolated. Its ease of destruction, except under strongly acidic conditions, may well account for this. As a colorless stable product, I·HCl may prove valuable in further studies tracing subsequent browning reaction intermediates.

NOMENCLATURE

Fructose-amino acids are 1-deoxy-1-amino-D-fructose derivatives of amino acids. Trivial names are employed for fructose-amino acids, *e.g.*, fructose-glycine, as suggested by Gottschalk (1952). Reduction products of fructose-amino acids are referred to as sugar alcohol-amino acids and the Amadori reaction product of D-xylose and glycine is designated as xylulose-glycine. The term "furacyl," as employed by Saldābols and Hillers (1955), designates the "furylcarbonylmethyl" group, which has been called the "furoylmethyl" group (Finot *et al.*, 1968). (The use of "furacyl" is analogous to use of "phenacyl" for "phenylcarbonylmethyl.") As is customary in the nomenclature of furans (Dunlop and Peters, 1953), the position of substitution on the ring is assumed to be "2" unless otherwise designated. Where appropriate, the 2 prefix is added for emphasis. Abbreviations used are FG, fructose-glycine; TCA, trichloroacetic acid; TFA-d, deuterated trifluoroacetic acid; TTC, triphenyltetrazolium chloride; furacyl bromide, bromomethyl-2-furyl ketone; HMF, 5-hydroxymethylfurfural.

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