Studies on the Role of 3-Deoxy-D-*erythro*-glucosulose (3-Deoxyglucosone) in Nonenzymatic Browning. Evidence for Involvement in a Strecker Degradation[1]

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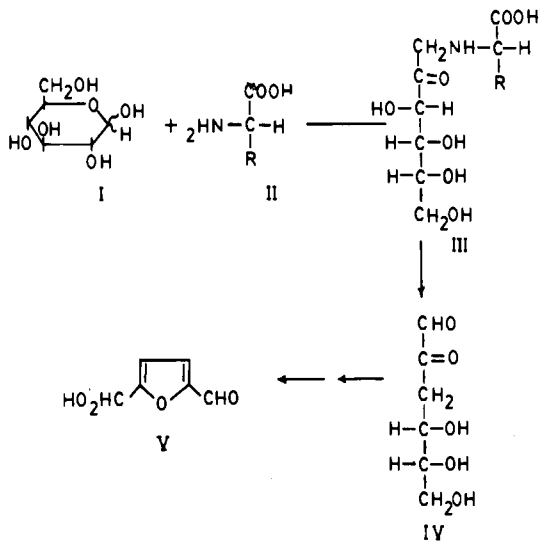
3-Deoxy-D-*erythro*-glucosulose (IV), a key carbohydrate-derived intermediate, is produced during the Maillard reaction. Its role in this reaction was studied with L-alanine and L-phenylalanine as representative amino acids. Measurements of rates of color formation, using IV, D-glucose, 5-(hydroxymethyl)-2-furaldehyde (HMF), and combinations thereof, in conjunction with L-alanine show that IV catalyzes color formation at a much faster rate than any other combination. NMR studies, using ^{13}C-labeled L-alanine (at both C-1 and C-2) in reactions with IV, indicate that no reactions leading to covalent bonds can be detected, such as Shiff bases or formation of stable adducts via reaction with the amino acid. Further experiments, using L-phenylalanine and IV clearly show that, for this system, the major degradative reaction involves a Strecker degradation, as evidenced by the appearance of phenylacetaldehyde, the expected product from a Strecker degradation reaction. It is thus concluded that IV, a documented intermediate in the Maillard reaction, serves, in part, as a reactant by participating in Strecker degradation reactions.

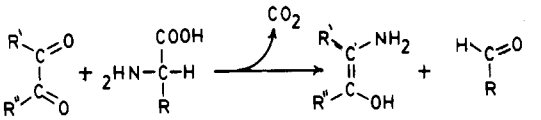
The Maillard reaction represents a complex series of degradative reactions involving carbonyl–amine interactions, ultimately leading to the production of polymeric pigments (nonenzymatic browning), the production of flavor and aroma constituents, and other degradative reactions. Typically, in foods, these reactions begin with sugars (as the carbonyl compound) and amino acids or proteins (as the source of the amine). During the early phase of such reactions (Scheme I), sugars (I) react with amino acids (II) to produce Amadori compounds (III). Amadori compounds are known (Feather and Russell, 1969) to undergo degradation at mild conditions and in acidic solution to dehydrate (with loss of the amine substituent) to 5-(hydroxymethyl)-2-furaldehyde (HMF, V) via the intermediate 3-deoxy-D-*erythro*-glucosulose (IV). Previous investigators (Anet, 1968; Kato, 1960) have reported the isolation and identification of IV from Amadori compounds and other systems, thus supporting the general view that it is an important intermediate in the Maillard reaction, representing the first dehydration product arising from D-glucose. Recently, a synthesis of IV was reported (Madson and Feather, 1981), thus permitting in-depth studies to be performed concerning its role and importance in the Maillard reaction.

The importance of IV in nonenzymatic browning is unclear. The possibility that a Strecker degradation (Scheme II) is involved in nonenzymatic browning has been suggested by several investigators (Olsson et al., 1981; Gottschalk, 1972). This reaction, which involves a dicarbonyl compound as a reactant, results in the production of carbon dioxide (arising from the carboxyl group of the amino acid), ammonia (from the α-amino group of the amino acid), and an aldehyde derived from the decarboxylated amino acid (Schonberg and Moubacher, 1952; Schonberg et al., 1948). It is noteworthy that IV could participate in such a reaction. There is a substantial body of data suggesting that a Strecker degradation may take

Scheme I



Scheme II



place during the Maillard reaction as part of the overall reaction scheme, even though the actual dicarbonyl participants have never been identified. Studies using isotopically labeled starting materials (Stadtman et al., 1952; Wolfrom et al., 1953; Feather and Huang, 1985) suggest that some (but not all) of the carbon dioxide produced during the reaction arises from C-1 of the amino acid. Other studies have shown that sugars, when reacted with amino acids, give rise to aldehydes (Holtermand, 1966). Other investigators (Olsson et al., 1978; Hodge, 1967) have suggested that many of the degradation products isolated and identified from sugar–amine reactions could have been produced as a result of a Strecker degradation.

In this paper, we report the preparation of IV and a study of its reactions with L-alanine and L-phenylalanine with respect to color formation, rates of disappearance

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from solution during reaction, and its role as a participant in Strecker degradation reactions.

EXPERIMENTAL SECTION

Materials and Methods. L-Alanine-*1*-^{13}C and -*2*-^{13}C (99.8 and 90.0 atom %, respectively) were obtained from MSD Isotopes, Rahway, NJ. NMR spectra were obtained on a Nicolet NT-300 FT spectrometer using broad-band decoupling and a ^{13}C frequency of 75.45 MHz. Optical spectra were obtained on a Varian Cary Model 210 spectrophotometer. Amino acids were obtained from U.S. Biochemical Corp., Cleveland, OH. Gas–liquid chromatography (GLC) was performed on a Varian 3700 instrument using a 25 m by 0.032 mm (i.d.) fused silica direct capillarry column containing 0.25-μm bonded methylphenyl (50%) silicone (Quadrex, New Haven, CT). Injector/detector temperatures were 300 °C, and peak areas were determined on a Shimadzu C-R3A integrator. Preliminary GLC runs were made on a Varian 1400 GLC equipped with a FID detector using a injector/detector temperature of 275 °C. For this instrument, standard 6-ft columns containing OV-17 as the liquid phase were used. The carrier gas was nitrogen at 20 mL/min. Mass spectra were collected on a GLC-interfaced CEC Model 21-110C mass spectrometer. The column used for the separation was the capillary column described above.

Preparation and Analysis of IV. Compound IV was prepared as described previously by Madson and Feather (1981). For analysis by GLC, IV was converted to the oxime and then trimethylsilylated as described by Mawhinney et al. (1980) for neutral sugars. These reactions were carried out in 3.0-mL reactivials (Pierce Chemical Co.). A known amount of mannitol was added as an internal standard. The GLC program for this separation (direct capillary column) was initial temperature 160 °C (2-min hold) followed by 4 °C/min to 215 °C. The carrier gas was helium at 3 mL/min. Analysis of D-glucose was carried out in the same manner with ribitol as the internal standard. Under these conditions, the oxime–TMS derivative of D-glucose appears as two peaks after 12 min and that of IV as two peaks after 9 min. Changes in concentrations of IV and D-glucose were calculated by comparing the peaks with those of the unreactive internal standard.

Analysis of Other Reactants. L-Alanine was converted to the *tert*-butyldimethylsilyl (*t*-BDMS) derivative as described by Mawhinney et al. (1986) with a known amount of glycerol as the internal standard. For this analysis, using the direct capillary column, the following program was used: initial hold at 100 °C (2 min), followed by a 10 °C/min rise to 280 °C. The carrier gas was helium at 5 mL/min. At these conditions, the alanine derivative had a retention time of 4.0 min and the glycerol of 10.0 min. Phenylacetaldehyde was converted to the oxime–TMS derivative as described by Mawhinney et al. (1980) for neutral sugars. For the GLC analysis on the direct capillary column, the following program was used: initial temperature 100 °C (2 min), followed by a rise of 4 °C/min to 260 °C. At these conditions, phenylacetaldehyde had a retention time of 9.0 min and appeared as two peaks.

Measurement of Rates of Color Formation. In a typical experiment, 0.1 M solutions of L-alanine and either IV, HMF, or D-glucose were made up in 0.1 M phthalate buffer (pH 3.5). Equal amounts (0.5 mL) of each reactant were added to a 6-mm glass tube sealed at one end. After addition, the other end was flame-sealed. Samples thus prepared were placed in a boiling water bath, and to collect a data point, a tube was removed, opened, and quantitatively diluted 10-fold with water and the color measured by direct measurement at 450 nm.

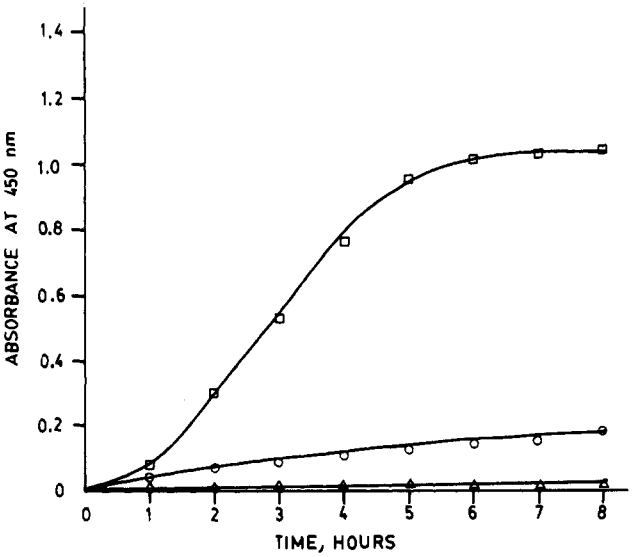

Figure 1. Rates of color formation for solutions of L-alanine and IV (□), D-glucose (△), and HMF (○). Initial concentrations of reactants are all 0.1 M.

Measurement of Rates of Loss of Reactants. In a typical experiment, 1-mL samples of combinations of reactants (all 0.4 M), in distilled water, were sealed in 6-mm glass tubes and incubated at 100 °C. Samples were removed at timed intervals, evaporated to dryness in a reactivial with a stream of dry nitrogen gas at less than 25 °C, and derivatized as described above, and the amount of unreacted starting material was determined by GLC as described above.

NMR Experiments. To 1.0 mL of deuterium oxide was added 120 mg of IV, 40 mg of ordinary L-alanine, 10 mg of L-alanine-*1*-^{13}C or -*2*-^{13}C, and 0.01 mL of dioxane (internal standard, δ 67.2). This solution, in a 5-mm spin tube, was incubated in a 100 °C water bath. At hourly intervals, up to 5 h, an NMR spectrum was collected for the reaction. Approximately 2500 scans were collected for each spectrum.

Identification of Phenylacetaldehyde. A solution (2.0 mL) of IV (15 mg) and L-phenylalanine (14 mg), in distilled water, was sealed in a glass tube and reacted at 100 °C for 0.5 h. The solution was then extracted three times with chloroform (2.0 mL for each extraction), the chloroform solution taken to dryness in a reactivial, and the residue converted to the oxime-TMS derivative as described above. This solution was then examined by GLC. Only two peaks were observed, corresponding in retention time to the oxime–TMS derivative of phenylacetaldehyde. Addition of an authentic sample of the oxime–TMS of phenylacetaldehyde caused an enhancement of the original peaks, but no new ones to appear.

RESULTS AND DISCUSSION

The results, with respect to color development in the model system studied, show (Figure 1) that IV clearly catalyzes color formation at a greatly enhanced rate as compared to D-glucose and HMF. This is consistent with the generally accepted fact that IV constitutes a highly reactive, but transient, intermediate produced during the Maillard reaction. Under the conditions of this experiment, neither D-glucose nor L-alanine alone gives rise to measurable color. The fact that IV is a dehydration product derived from D-glucose (minus 1 mol of water) suggests that IV, or a dehydration product derived from it, may well constitute the repeating unit of Maillard polymers, at least in the early stages of the reaction.

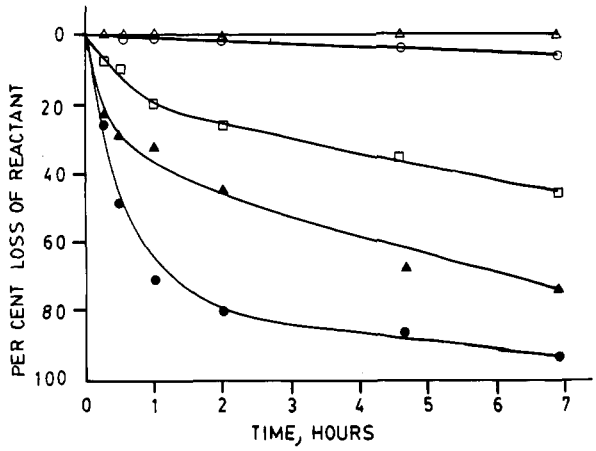

Figure 2. Disappearance from solution of various reactants: L-alanine (in the presence of D-glucose) (△); D-glucose (in the presence of L-alanine) (○); L-alanine (in the presence of IV) (□); IV (alone) (▲); IV (in the presence of L-alanine) (●). Initial concentrations of reactants are all 0.4 M.

Figure 2 shows some data on rates of disappearance of various reactants from solution during a Maillard reaction. As expected, the D-glucose–L-alanine system is relatively stable when compared to IV and L-alanine, or IV alone. It is also apparent that L-alanine has a significant effect on the rate of disappearance of IV (and vice versa), suggesting an interaction of some type.

In order to probe the nature of the interaction between IV and L-alanine, some NMR experiments were undertaken. Roper et al. (1983) prepared a number of Amadori compounds produced from D-glucose and a variety of amino acids and have made the assignments for all the carbon signals for these compounds. Because of the great sensitivity of carbon-13 resonances to their chemical environment, these workers found that a carbohydrate residue, when it is covalently bonded to the α-amino group of the amino acid, causes a measurable shift for the resonances of the C-1 and C-2 carbons of the amino acid. In a recent study of the interaction of D-glucose and glycine-*1*-^{13}C and -*2*-^{13}C (90 atom %) we were able to verify this and to detect low levels of Amadori compounds in the solution by use of this technique (Feather and Nelson, 1984).

For this study, IV was incubated with L-alanine-*1*-^{13}C (99 atom %) and -*2*-^{13}C (90 atom %), in separate experiments, as described in the Experimental Section. Prior to reaction, the spectra for both solutions showed only singlet signals, corresponding to the resonances for C-1 (δ 176.4) and C-2 (δ 51.3). Spectra were recorded periodically, for up to 5 h of reaction time. No new signals were observed indicative of a reaction product of any type. This suggests that if a reaction occurs between IV and L-alanine at the α-amino group, such as Shiff base formation or the formation of a stable adduct via reaction of IV with the amino acid, the product produced does not accumulate to any measurable extent during any phase of the reaction. These findings are also consistent with a reaction involving a Strecker degradation.

In order to examine the latter possibility, some experiments were undertaken using IV and L-phenylalanine as reactants. The latter amino acid was chosen because the Strecker degradation product expected is phenylacetaldehyde, a substance having a low volatility and, hence, much more easily identified and detected than acetaldehyde (the expected product arising from L-alanine itself). Chloroform extracts of the reaction mixture gave a compound that, after conversion to the oxime–TMS derivative, was identical (by GLC and MS) to authentic standards. Major mass spectral peaks were found at m/z 207, 192, 116, 103, 91, 89, 77, and 65.

The data collected strongly suggest that an important role of IV, in addition to its serving as an intermediate in dehydration reactions leading to furan derivatives, and as a major participant in color formation, is to additionally participate in a Strecker degradation reaction, thus catalyzing the degradation of amino acids during the reaction and, indirectly, being responsible for many of the aldehydes associated with the Maillard reaction.

ACKNOWLEDGMENT

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Registry No. HMF, 67-47-0; alanine, 56-41-7; phenylalanine, 302-72-7; glucose, 50-99-7.

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Lipoxygenase in Fish Tissue: Some Properties of the 12-Lipoxygenase from Trout Gill

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The gill and skin tissues from several fish species contain active lipoxygenase, which is capable of oxidizing polyunsaturated fatty acids into hydroperoxides. Gill lipoxygenase of rainbow trout exhibited similar reactivities toward arachidonic, eicosapentaenoic, and docosahexaenoic acids, but low reactivity toward linoleic acid. The lipoxygenase exhibited activity from pH 7 to 9 with optimum pH at 7.5. The enzyme was rapidly inactivated at temperatures above 40 °C. Stability of gill lipoxygenase was enhanced in the presence of glutathione. Inactivation of lipoxygenase by sulfhydryl-specific reagents suggested that thiol groups were involved in its activity.

The demand for high-quality fish and seafood products should increase because of their apparent beneficial effects on health (Herold and Kinsella, 1986). However, the high degree of unsaturation of fish lipids makes them susceptible to oxidation and quality deterioration especially if fish is not handled properly. The problem of quality deterioration in fish is closely related to oxidative instability of lipids (McDonald et al., 1979). To control or minimize lipid oxidation, more basic information concerning the initiation mechanism of lipid oxidation is needed.

When fish is killed and tissue damaged, certain enzymes such as lipoxygenase of fish gill and skin (German and Kinsella, 1985, 1986a), peroxidase of fish blood (Kanner and Kinsella, 1983), and microsomal NADH peroxidase of fish muscle (Slabyj and Hultin, 1984) may become uncontrolled and initiate lipid peroxidaton. Lipoxygenase is present in gill and skin tissues of fish and capable of initiating oxidation of polyunsaturated fatty acids to produce unstable hydroperoxides (German and Kinsella, 1985, 1986a). These hydroperoxides, following carbon–carbon cleavage at the hydroperoxide group, are potential precursors of many compounds, such as hexanal, 4-heptenal, and 2,4-heptadienal. These carbonyls are sources of oxidative off-flavors that can adversely affect taste and smell of fish (Josephson et al., 1984).

Because of its potential role in generating oxidative off-flavors in fish, the properties of lipoxygenase as they affect fish qualities are of practical interest. Recently we observed lipoxygenase in trout gill and skin tissues (German and Kinsella, 1985, 1986a). The present study was undertaken to determine the presence and relative activities of lipoxygenase in gill and skin tissues of several species of fresh water fish. Furthermore, because its activity and concentration is high in trout gill tissue, we used gill lipoxygenase to determine properties (the optimum pH, substrate specificity, heat stability, enzyme self-inactivation, and the effects of thiol reagents on lipoxygenase activity) of this lipoxygenase.

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MATERIALS AND METHODS

Materials. Linoleic (18:2, $n-6$), arachidonic (20:4, $n-6$), eicosapentaenoic (20:5, $n-3$), and docosahexaenoic (22:6, $n-3$) acids were obtained from Nu-Chek Prep (Elysian, NY). Radioactive [1-^{14}C]arachidonic, [1-^{14}C]eicosapentaenoic, [1-^{14}C]docosahexaenoic, and [1-^{14}C]linoleic acids were purchased from New England Nuclear (Boston, MA). Glutathione, iodoacetamide, and *p*-chloromercuribenzoate (pCMB) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl acetate was obtained from Fisher Scientific Co. (Rochester, NY). Methanol was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Chloroform and glacial acetic acid were from Mallinckrodt Inc. (St. Louis, MO).

Lipoxygenase Preparation. Samples of gill tissue (1 g) were carefully excised from young freshly killed rainbow trout (15 g) (Tunison Fish Laboratory, Cortland, NY). The gill tissue was homogenized in 40 mL of 0.05 M pH 7.4 phosphate buffer with 1 mM glutathione (*r*-L-glutamyl-L-cysteinylglycine) on a Polytron homogenizer. The homogenate was centrifuged for 15 min at 15000*g* at 4 °C. The resultant supernatant fraction was used as the crude enzyme source without further purification. The enzyme preparation was either used immediately or frozen in liquid nitrogen as droplets and stored at −70 °C until use. Protein concentration was estimated by using phenol reagents with bovine serum albumin as standard (Lowry et al., 1951).

Skin tissues (1 g) were carefully excised from young freshly killed rainbow trout, and residues of muscle on the skin were completely removed. They were then cut into small pieces to facilitate homogenization. The skin homogenate was then centrifuged, and protein concentration was determined as stated above.

Lipoxygenase Assay. To assay lipoxygenase activity, the enzyme preparation (1 mg of protein/mL) was incubated at 25 °C with a polyunsaturated fatty acid such as arachidonic acid (50 μM) using labeled [1-^{14}C]eicosatet-

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