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Studies on the Mechanism of Formation of 4-Hydroxy-5-methyl-3(2*H*)-furanone, a Component of Beef Flavor, from Amadori Products

Kevin B. Hicks and Milton S. Feather*

1-Deoxy-1-dibenzylamino-D-fructuronic acid (**2**) was found to readily dehydrate at 100° and pH 7 to 4-hydroxy-5-methyl-3(2*H*)-furanone (**1**). When **1**, labeled with ^{14}C at C-1 of the hexuronic acid, was used as a starting material, **1** was produced exclusively labeled on the methyl group. On performing the reaction in deuterium oxide solution, **1** was produced having essentially all the methyl protons exchanged with deuterium. In 2 *N* sulfuric acid **2**, 1-benzylamino-1-deoxy-D-fructuronic acid (**3**), and 1-benzylamino-1-deoxy-D-xylulose (**4**) all gave rise to 2-furaldehyde. With the exception of **4**, all gave rise to reductic acid (2,3-dihydroxy-2-cyclopenten-1-one) as well. At pH 7, all three produced **1** as the major dehydration product. At pH 2.5, **1** was observed as a major product from **2**, while it was produced in yields of 6% or less from **3** and **4**.

There is abundant indirect evidence (Hodge et al., 1963) which indicates that a number of flavor and aroma constituents found in food preparation are produced during the cooking process by the condensation of carbohydrates with basic amino groups to form Amadori compounds (1-amino-1-deoxy-2-ketoses) and their subsequent dehydration products. Thus, both maltol (3-hydroxy-2-methylpyran-4-one) and isomaltol (3-hydroxy-2-acetylfuran) which contribute to the flavor of, and are found in baked breads, can be produced synthetically from Amadori compounds (Hodge and Nelson, 1961). 4-Hydroxy-5-methyl-3(2*H*)-furanone (Figure 1), a constituent of beef broth, has also been reported produced from Amadori compounds. This furanone has been prepared by heating D-xylose (Severin and Seilmeier, 1968), D-ribose (Peer et al., 1968a) and D-ribose 5-phosphate (Peer et al., 1968b) with amine salts. The compound has a caramel-like or burnt aroma and its isolation from beef broth (Tonsbeek et al., 1968) indicates that it is a constituent of cooked beef flavor. In an earlier communication from this laboratory, we reported that this furanone is also produced during the decomposition of 1-deoxy-1-dibenzylamino-D-fructuronic acid (Hicks et al., 1974) which was synthesized by the condensation of D-glucuronic acid with dibenzylamine, thus showing that hexuronic acids can also serve as a source of this material during the cooking process.

The present study was aimed, in part, at elucidating aspects of the mechanism for formation of the furanone from an Amadori compound derived from D-glucuronic acid. These studies involved isotopic tracer experiments and deuterium incorporation measurements.

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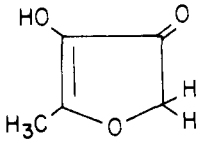

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Figure 1.

A second purpose of the study was to determine what qualitative effects acidity of the dehydration medium and amine basicity had on yields of three of the expected dehydration products arising from Amadori compounds of D-xylose and D-glucuronic acid. The observed facts were examined to see if they were consistent with previously proposed mechanisms for the dehydration of 1-amino-1-deoxy-2-ketoses.

EXPERIMENTAL SECTION

Materials and Methods. Nuclear magnetic spectra were run on a Varian T-60 spectrometer. Ultraviolet spectra were obtained using a Coleman Model 124 double beam spectrophotometer and scintillation counting was done on a Packard Model 3003 Tri-Carb instrument using Bray's (1960) solution as the scintillant. In all cases, an internal toluene-^{14}C standard was used to calculate efficiencies. Thin-layer chromatography was run on silica gel HF-254 (Brinkmann) using the following irrigants: (A) chloroform–acetic acid (9:1); (B) chloroform–methanol (9:1); (C) ethyl acetate–benzene (2:98); (D) 2-propanol–acetic acid (9:1); and (E) methanol. Developed plates were visualized using ultraviolet light.

Gas chromatography was performed on a Varian Model 1400 chromatograph using a 5-ft column packed with 3% GC SE-30 on 100–120 mesh Varaport 30. For mass spectral measurements, this column was interfaced with a CEC Model 21-110 C mass spectrometer.

Preparation of Amadori Compounds. Both 1-deoxy-1-dibenzylamino-D-fructuronic acid (**2**) and 1-benzylamino-1-deoxy-D-fructuronic acid (**3**) were prepared using published methods (Heyns and Baltes, 1960). Compound **2** was obtained as a crystalline solid having mp 150° (lit. mp 150°) and compound **3** as a thick yellow syrup. The oxalate salt of 1-benzylamino-1-deoxy-D-xylulose (**4**) was prepared according to Micheel and Hagemann (1959) and had mp 135° (lit. mp 138–140°).

Preparation of 4-Hydroxy-5-methyl-3(2*H*)-furanone (1). In a typical experiment, 10 g of **2** was dissolved in 180 ml of 2 *N* acetic acid and the solution, which had a pH of 6.8, was refluxed for 1 hr. After cooling to 25°, the solution was passed through a column of Dowex 50 (hydrogen form, 200 ml) and the effluent extracted three times with chloroform. After drying over anhydrous sodium sulfate, the chloroform was evaporated to dryness giving crystalline **1**. The 1 so produced was purified by sublimation at 60° and 0.25 mm, mp 129°.

Preparation of D-Glucuronic Acid-*1*-^{14}C. This compound was prepared by converting D-glucose-*1*-^{14}C to 1,2-*O*-isopropylidene-D-glucofuranose-*1*-^{14}C (Schmidt, 1963) and oxidation of the C-6 hydroxymethyl group to a carboxyl group using platinum on charcoal as the catalyst and oxygen as the oxidant (Marsh, 1952). After hydrolysis, the resulting compound was crystallized as D-glucuronolactone-*1*-^{14}C from water–methanol to constant radiochemical activity. For conversion of the lactone to the free acid, it was dissolved in water and an equivalent amount of sodium hydroxide was slowly added. The resulting solution of sodium D-glucopyranuronate-*1*-^{14}C was then passed through a column of Dowex 50 (hydrogen form) and the effluent evaporated to a syrup at 40° in vacuo from which D-glucuronic acid-*1*-^{14}C readily crystallized after seeding.

Preparation and Degradation of 1-^{14}C. A 25-g sample of D-glucuronic acid-*1*-^{14}C having a specific activity of 0.27 μCi/mmol was converted to compound **2** and thence to **1** as described above. The furanone-^{14}C was dried and then sublimed to constant radiochemical activity and melting point. A 50-mg sample of 1 was then converted to carbon dioxide, water, and acetic acid by oxidation with chromium trioxide (Maciak, 1962). The resulting acetic acid was then quantitatively distilled from the solution and the distillate made up to 50 ml. Several 2.0-ml aliquots were used to determine the radiochemical content, and 2–10-ml aliquots were titrated with standard 0.0200 *N* sodium hydroxide solution to determine the acetic acid content. Specific radiochemical activities were calculated from the data obtained.

Deuterium Exchange Experiment. Approximately 10 g of **2** was dissolved in 160 ml of 98% deuterium oxide and the solution adjusted to pH 6.8 with 20 ml of acetic acid. After heating at 100° for 1 hr, the **1** so produced was isolated as described above and purified by sublimation. NMR spectra were scanned and integrated at a concentration of 40 mg/ml in deuteriochloroform.

Influence of Acidity on Product Formation. For studies on 2-furaldehyde formation, approximately 0.5-g samples of compounds **2, 3,** and **4** were placed in flasks containing 25 ml of 2 *N* sulfuric acid. The solutions were brought to boiling and the distillate was collected until approximately 80% of the solution had been distilled. After diluting to a convenient volume, a portion of each distillate was subjected to an ultraviolet scan in the region 200–300 nm and phenylhydrazine was added to the remaining portions. The precipitated 2-furaldehyde phenylhydrazones were recrystallized from methanol to constant melting point.

To determine production (in strong acid) of furanone, reductic acid, and in the case of hexuronic acid derivatives, 5-formyl-2-furoic acid, approximately 2-g samples of **2, 3,** and **4** were heated at 100° for 45 min in 25 ml of 2 *N* sulfuric acid. After cooling, each solution was neutralized with barium carbonate and filtered through Celite and the filtrate percolated through a column containing 100 ml of Dowex 50 (hydrogen form). On evaporation in vacuo at 40°, the residue was subjected to thin-layer chromatography using irrigants A, D, and E. Preparative thin-layer chromatography using solvent A was then used to isolate materials for ultraviolet absorption spectrophotometry.

For experiments at pH 7, approximately 4-g samples of **2, 3,** and **4** were heated at 100° for 1 hr in 50 ml of pH 7 buffer. After cooling, the solutions were deionized using a column containing Dowex 50 (hydrogen form) and the effluent extracted 10 times with chloroform. After drying over anhydrous sodium sulfate, the chloroform was evaporated to dryness and the residue examined by thin-layer chromatography in irrigants A and B. For isolation of furanone for mass spectral data collection, preparative thin-layer chromatography using irrigant A was used. For experiments at pH 2.5, approximately 0.2-g samples of **2, 3,** and **4** were dissolved in 5 ml of 2 *N* acetic acid, and, when necessary, the pH was adjusted to pH 2.5 by the dropwise addition of glacial acetic acid. After heating for 1 hr at 100°, 2 μl samples of the solutions were spotted on thin-layer chromatographic plates developed with solvent A.

RESULTS

4-Hydroxy-5-methyl-3(2*H*)-furanone (**1**) was prepared from 1-deoxy-1-dibenzylamino-D-fructuronic acid by treatment of an aqueous solution at pH 6.8 at 100° as was described in an earlier communication (Hicks et al., 1974) which, in addition, described its physical and chemical characterization. In this work, 1-benzylamino-1-deoxy-D-fructuronic acid and 1-benzylamino-1-deoxy-D-xylulose were also prepared, and, on treatment at 100° and pH 7, were also found to give rise to isolable amounts of the furanone which was identified from its thin-layer chromato-

graphic flow rate, its ultraviolet spectrum, which showed λ_{max} 284 nm (water), and its mass spectrum all of which were identical with an authentic standard.

In order to examine the pathway of carbon for the reaction, D-glucuronic acid-*1*-^{14}C was prepared from D-glucose-*1*-^{14}C and the resulting uronic acid was converted to 1-deoxy-1-dibenzylamino-D-fructuronic acid-*1*-^{14}C which had a specific activity of 0.027 μCi/mmol. Dehydration of this material yielded 1-^{14}C with a specific activity of 0.026 μCi/mmol. The furanone-^{14}C was then subjected to a Kuhn–Roth oxidation, a process which permits isolation of the 5-methyl carbon atom as acetic acid. The resulting acetic acid had a specific activity of 0.024 μCi/mmol.

In order to examine the relevance of certain proposed intermediates in such reactions, a sample of the furanone was prepared from **2** in deuterium oxide solution, and the amount of incorporation of carbon-bound deuterium was determined by comparing integrated nuclear magnetic resonance spectra of this material with an ordinary sample. Carbon-bound proton signals were observed at δ 2.28 (methyl group) and 4.65 (position 2) in the ratio of 3:2 for the ordinary compound. The sample prepared in deuterium oxide showed a ratio of approximately 0:1. In a parallel experiment, designed to examine whether this incorporation was a result of an equilibration of reaction intermediates or product with solvent, a sample of furanone was treated with acidified deuterium oxide at the conditions in which it was formed. The results showed that protons at position 2 readily exchanged while those on the methyl group did not. Thus, the exchange of *C*-methyl protons with solvent occurs during the formation of reaction intermediates and not after product formation.

In order to test the effects of acidity on product formation, three Amadori compounds, **2, 3,** and **4,** were prepared and the resulting products produced during their degradation at pH 7, in 2 *N* sulfuric acid, and at pH 2.5 were examined. On treatment with 2 *N* sulfuric acid for 45 min, reductic acid (2,3-dihydroxy-2-cyclopenten-1-one) was detected as a product from **2** and **3,** as evidenced by thin-layer chromatography and ultraviolet spectra, while none could be detected from **4.** Only from compound **3** could furanone be detected as evidenced by thin-layer chromatography. In a further experiment, all three materials were heated over a 3-hr period in 2 *N* sulfuric acid and slowly distilled during the heating period. In all cases, 2-furaldehyde was identified as a reaction product in the distillate as evidenced from its ultraviolet spectrum (λ_{max} 278 nm) and isolation as the crystalline phenylhydrazone (mp 94°). Yields, as measured spectrophotometrically, were 16% for **2,** 23% for **3,** and 41% for **4.**

All three Amadori compounds were dissolved in 2 *N* acetic acid and the pH adjusted to 2.5 where necessary with glacial acetic acid. After heating at 100°, furanone was readily detected from **2** by thin-layer chromatography, but none was observed from **3** or **4** at these conditions. Only in pH 2.5 experiments were the reaction mixtures not taken to near dryness before thin-layer analysis. Instead, at this pH, 2 μl samples were withdrawn from the reaction mixture and then analyzed. From previous studies on minimum detectable levels of the furanone in thin-layer work, it was determined that since **3** and **4** gave no observable sign of furanone, at the stated experimental concentrations, it must have been produced, if at all, in yields less than 6%.

At pH 7, all three compounds gave rise to furanone. In all cases, it was identified by comparison of its mass spectrum, ultraviolet spectrum, and its thin-layer chromatographic flow rates with those of an authentic sample.

DISCUSSION

In strongly acidic solution, both pentoses and hexuronic acids give rise to both 2-furaldehyde and reductic acid (Feather and Harris, 1973). Both of these dehydration products arise from hexuronic acids by decarboxylation. In addition, 5-formyl-2-furoic acid is usually observed as a minor dehydration product of hexuronic acids (Feather and Harris, 1966). That the Amadori compounds produced from both D-xylose and D-glucuronic acid give rise to furanone is therefore not surprising in this respect, since dehydration products common to both sugars are normally observed. Hodge et al. (1972) have recently discussed possible mechanisms of formation of methylfuranones and related compounds in foods and from Amadori compounds. It has been generally thought that the Amadori compound (**5**) (Figure 2) undergoes 2,3-enolization to the 2,3-enediol (**6**) and is thence converted by the elimination of the amino group to the enolic form (**7**) of a 1-deoxy-2,3-dicarbonyl intermediate (**8**) which subsequently further dehydrates to flavor and aroma constituents. Anet (1964) has discussed probable factors which control the 1,2-enolization of an Amadori compound vis-a-vis the 2,3-enolization. It is generally concluded that 1,2-enolization is promoted by a sufficiently strongly acidic environment to cause protonation of the nitrogen atom, and that the reaction products are 2-furaldehydes or compounds mechanistically related to them, such as reductic acid. For 2,3-enolization to occur, the Amadori compound should be partially unprotonated giving rise to a form which would discourage 1,2-enolization and, therefore, indirectly favor 2,3. These predictions are verified in this study. In strong acid, the Amadori compounds studied all produced 2-furaldehyde and, with the exception of **4,** reductic acid, both of which presumably arise from the initial 1,2-enolization of the Amadori compounds. At these conditions, **1** was either undetectable or found in trace amounts. However, at pH 7, all three compounds gave **1** as the major identifiable product. At pH 2.5, **1** was produced in major amounts only from **2.** The fact that **1** is produced from **2** in much larger yields than from **3** or **4** at these conditions is presumably related to the basicity of the Amadori compound. Compound **2,** a tertiary amine, would be expected to be a weaker base than either **3** or **4,** both of which are secondary amines.

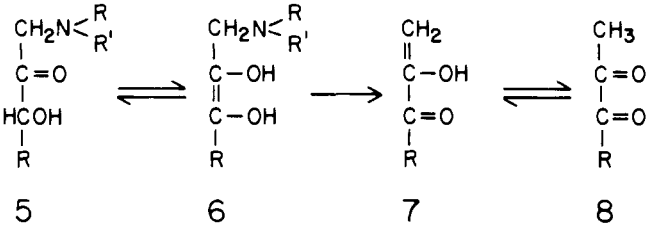

Figure 2.

The ^{14}C tracer experiment, which shows that C-1 of the hexuronic acid corresponds to the 5-methyl group of the furanone, is also consistent with a 1-deoxy-2,3-dicarbonyl intermediate such as **8** and also indicates that the furanone is a decarboxylation product. These data are also similar to those obtained by Peer et al. (1968a) for the conversion of D-ribose-*1*-^{14}C to the furanone in the presence of secondary amine salts.

The deuterium exchange data clearly indicate that solvent exchange of deuterium with *C*-methyl protons occurs during the reaction and not via an equilibration of product with solvent. These data are consistent with the mechanism proposals, i.e. an extensive equilibration of intermediates **7** and **8** (Figure 2) during the reaction.

The fact that deuterium incorporation observed at position 2 could occur either during the reaction or via an equilibration of furanone after its formation does not permit these data to be used in a mechanism interpretation. Thus, these data are of no value in determining the exact step for the decarboxylation. It is noteworthy, however, that the complete dehydration of such hexuronic acid de-

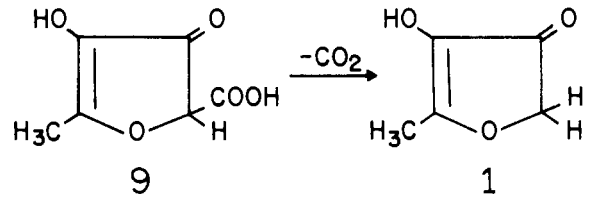

Figure 3.

rived Amadori compounds could give rise to **9** (Figure 3) which is a β-keto acid and would be expected to readily decarboxylate to the furanone (**1**) incorporating one solvent deuterium atom in the process.

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Melanoidins and Carbohydrates in Roasted Barley

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Carbohydrate constituents of barley have been determined. Changes occurring during the process of roasting, mainly the interaction between carbohydrates and amino compounds, have been studied. A brown amorphous substance of melanoidin type was isolated by column chromatography on ion-exchange resin Permutit ES. Its simplest empirical formula of $C_{18}H_{27}O_{11}N$ was determined by elementary analysis which was compared to a model substance obtained from reaction between L-aspartic acid and D(+)-glucose at 93° for 230 hr. The empirical formula of the melanoidin model substance was found to be $C_{18}H_{26}O_{11}N$. Both ir and uv spectra of melanoidins isolated from roasted barley and model substance were found to be similar. A method for determination of melanoidins by spectrophotometry at 430 nm was developed.

The changes of constituents during the roasting of cereals have been largely concerned with the changes in concentration of nonstructural carbohydrates, which amounts to over 60% in unroasted grain. Underwood and Deatherage (1952) found that the concentration of water-soluble constituents, primarily nonstructural carbohydrates, decreases during the process of roasting. On the other hand, some simple constituents are formed as shown by a positive test for carbohydrates of glucose and saccharose type. The monosaccharides were changed during the process of roasting so that one part, in reaction with proteins and amino acids, produced complexes of melanoidin and the other part was lost by decarboxylation and dehydration. Wolfrom et al. (1960) reported that holo- and hemicellulose have been slightly decreased during the roasting of coffee beans. Knauf et al. (1941) reported that anhydromannose in defatted palm seeds was degraded into a soluble form of 1,6-anhydro-α-D-mannopyranose by pyrolysis during the process of roasting.

Roasting of cereals at a temperature of 200° is inevitably followed by the formation of brown pigments of melanoidin type (Holtermand, 1966; Reynolds et al., 1962; Anet, 1960, 1961, 1962; Burton and McWeeny, 1964). Wolfrom (1945) found that the sugar-C:N:methylene-C ratio must be 1:1:1 for the optimal production of melanoidins while Anet (1961, 1962) has shown that the ratio of sugar-C:N may be 6:1. This confirms that melanoidins can be of different compositions from various sources (Grujić-Injac et al., 1971). Kass and Palmers (1940) state that the formation of brown pigments at high temperatures does not allow the interpretation of the "interaction" between amino acids and reducing sugars as a bifunctional reaction. In fact, this "interaction" is complex and unidentified. Formation of melanoidins is due to caramelization of sugar and adsorption of aldocaramel on colloidal particles of protein.

In the preparation and processing of foods, one is soon acquainted with the phenomenon of browning associated with heated and stored products. These reactions do not require enzymic catalysis and are referred to as nonenzymic. Many food industries are directly concerned with the production of these brown products, to the extent that they contribute to the flavor, color, and aroma of their products, e.g., coffee, caramel, bread, and breakfast cereals. However, even in these processes careful control must be exerted to prevent excessive browning, which could lead to unpleasant changes occurring in the food product.

This study was undertaken to investigate the influence of applied temperature on food processing. It was interesting to study both the concentrations and changes of carbohydrates and amino acids during the roasting of barley

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