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Role of Phosphate and Carboxylate Ions in Maillard Browning

GEORGE P. RIZZI*

Procter & Gamble Company, Winton Hill Business Center, 6071 Center Hill Road, Cincinnati, Ohio 45224

The Maillard reaction of carbohydrates and amino acids is the underlying chemical basis for flavor and color formation in many processed foods. Phosphate and other polyatomic anions will accelerate the rate of Maillard browning, and this effect has been explained by invoking enhanced proton abstraction from intermediate Amadori compounds. In this work, the effect of phosphate and carboxylate ions on browning was measured for a series of reducing sugars with and without the presence of β-alanine. Significant browning was observed for sugars alone suggesting that polyatomic anions contribute to Maillard browning by providing reactive intermediates directly from sugars. A mechanism is proposed for decomposition of sugars by polyatomic anions and efforts to trap reactive species using *o*-phenylenediamine (OPD) are described. The results of this study suggest how complications may arise from the popular usage of phosphate buffers in the study of Maillard reaction kinetics. In addition, the results imply how phosphates may be useful for enhancing browning during food processing.

KEYWORDS: Maillard; browning; phosphate ion; carboxylate ion; catalysis; mechanism

INTRODUCTION

The Maillard reaction is a long-recognized source of flavor and color in processed foods. Today, although much has been learned about Maillard chemistry, the effective control of the reaction during food processing remains elusive. In a recent review, Martins et al. (*1*) summarized Maillard chemistry and pointed out how multiresponse kinetic modeling might be applied to control food quality attributes resulting from the reaction. A possible addition to improve this promising approach is to incude a parameter relating to the effects of catalytic agents on browning. Toward this end, we wish to report our observations on the catalytic role of phosphate and carboxylate ions in Maillard browning.

The accelerating effect of phosphate on Maillard browning is well-known and was first studied in detail by Reynolds (*2*). Reynolds observed that no inorganic phosphate was consumed during the reaction and that no stable intermediate containing covalently bound phosphorus could be detected. A more extensive mechanistic study of phosphate catalysis based on kinetic data was reported by Potman and van Wijk in 1989 (*3*). From a pH study, it was concluded that the catalytic species is the dihydrogen phosphate ion that was presumed to act as a base for catalyzing the Amadori rearrangement. In concert with this argument, phosphate was recently shown to accelerate the degradation of an Amadori compound into an amino acid and the parent sugar (*4*). However, despite these results, the exact mechanism for phosphate involvement is still not clear. For example, Bell (*5*) had later concluded that phosphate acts as a bifunctional (acid/base) catalyst to speed up the glycosylation of amino acids.

The independent reaction of sugars with phosphate has not received much attention as a possible contributor to Maillard browning. Potman and van Wijk (*3*) observed that the rate of glucose loss in a glycine/glucose reaction was dependent on phosphate concentration, but the effect of this phenomenon on browning was not considered. In a related mechanistic study, Weenen and Apeldoorn (*6*) observed low yields of sugar cleavage products, glyoxal and pyruvaldehyde in sugar/phosphate reactions without amino acids.

EXPERIMENTAL SECTION

Materials. All chemical reagents were high quality commercially available materials. Sugars were D-isomers except for L-rhamnose. Phosphate buffers were prepared by combining equimolar aqueous solutions of Na_2HPO_4 and NaH_2PO_4. Bisulfite/sulfite buffer was obtained by adding NaOH to sodium bisulfite solution. Bis/tris buffer consisted of 0.10 M aqueous 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol adjusted to ca. pH 7 with 6 N HCl. Succinate and citrate buffers (ca. pH 7) were obtained by partial neutralization of 0.10 M aqueous succinic or citric acid with NaOH. Acetate buffer was aqueous, 0.10 M sodium acetate (pH 7.80). TLC was usually done on 0.25-mm silica gel G 60 (F_{254}) plates eluted with 95:5 v/v CH_3CN-water solvent, and analytes were visualized with ultraviolet light. For preparative TLC, visualized bands were scraped from the plates with a razor, and compounds were extracted with methanol. Reversed-phase TLC was done using HP-TLC, RP-18 (F-254-S) plates (EM Reagents, Merck), with 90:10 v/v MeOH−water solvent.

General Reaction Procedure. Maillard browning reactions were usually performed by refluxing 0.10 M aqueous buffer solutions

* To whom correspondence should be addressed. George P. Rizzi, 542 Blossomhill Lane, Cincinnati, OH 45224-1406. Tel. (513) 761-0816. E-mail: georgerizzi@prodigy.net.

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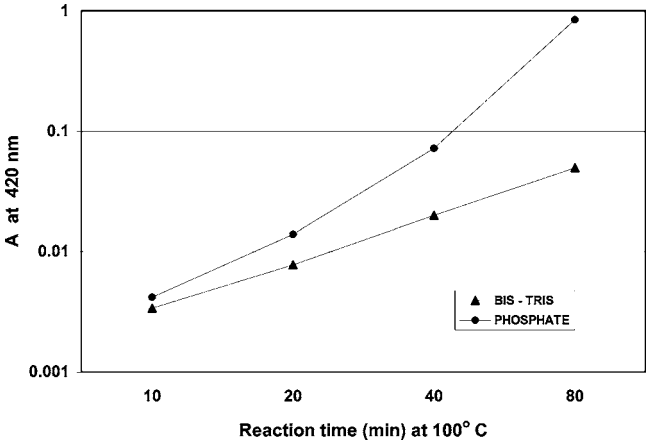


Figure 1. Absorbance at 420 nm vs time for xylose/β-alanine reactions in phosphate and bis–tris buffer solutions at pH 7.3.

containing various carbohydrates (0.10 M) and β-alanine (0.033 M) in two-necked round-bottomed flasks fitted with a rubber septum for withdrawal of samples. Reactions were performed at approximately neutral pH under atmospheric pressure at ca. 100−105 °C for periods ranging up to 300 min. At increasing time intervals, small samples of hot reaction mixtures were withdrawn with pre-chilled syringes and immediately diluted 1:4 with 23 °C water prior to absorbance measurement (vs pure water) in 1-cm cuvettes at 420 nm.

Preparation and Analysis of Quinoxaline Derivatives. Following an 80-min reaction, ribose/phosphate buffer mixtures (60 mL) were rapidly cooled to 25 °C, treated with *o*-phenylenediamine (OPD, 100 mg) and incubated at 50 °C for 30 min. Quinoxalines and unreacted OPD were extracted with CH_2Cl_2 and concentrated for GC/MS analysis or separation by prep TLC. Volatile quinoxaline and methylquinoxalines were identified by their GC retention times (R_t) and MS of available standards. 2-Ethylquinoxaline was tentatively identified by its library MS spectrum alone. Nonvolatile quinoxalines were isolated by prep TLC and tentatively identified through literature R_f, NMR, and UV data. Addition of OPD at the start of ribose−phosphate buffer reactions produced somewhat higher yields of the same quinoxalines in a similar ratio. No evidence for quinoxaline products was found in control experiments run in bis−tris buffer.

Instrumental Analyses. UV−vis data were obtained with a computer interfaced Hitachi U-3010 spectrophotometer. For GC/MS, a Hewlett-Packard HP5973 MSD and HP6980 GC with a 30 M (0.25 mm) fused silica column (1 μm DB-5 MS) were used. 300-MHz proton NMR data were obtained in $CDCl_3$ solution using a Varian INOVA-300 spectrometer. The external reference standard was the $CDCl_3$ signal at 7.26 ppm.

RESULTS AND DISCUSSION

Effects of Various Buffers on Browning. Maillard browning was followed by measuring changes in absorbance (A_{420}) versus time in 0.10 M buffer solutions at reflux temperature (ca. 100 °C), initially containing reducing sugars (0.10 M) and β-alanine (0.033 M). β-Alanine was chosen as the common amino acid to minimize complications resulting from competitive Strecker degradations. Reactions were generally monitored during 160 min when visual color ranged from colorless to shades of yellow to orange and finally to red. Absorbance at 420 nm appeared to increase exponentially with time, and absorbance data were conveniently analyzed in semilogarithmic plots, (e.g., **Figure 1**). In **Figure 1**, enhanced browning due to phosphate is observed in a xylose/β-alanine reaction when compared with the same reaction run in the relatively inert bis−tris buffer. In **Table 1**, absorbances are shown at a single reaction time (80 min) for comparisons. Replicate experiments typically exhibited absorbance precision ranging from ± 3% to ± 20% with the lowest absorbances showing the greatest error.

Table 1. Browning of Sugars with or without β-Alanine in 0.10 M Bis–Tris and Phosphate Buffers at Initial pH 7.3 ± 0.04

	bis–tris[b]		phosphate[b]	
sugar[a]	A_{420}	Δ pH	A_{420}	Δ pH
ribose	.061	–0.17[c]	1.17	–0.86[c]
ribose-no β-ala	.003	–0.16	0.262	–0.43[c]
xylose	.050	–0.09	0.837	–0.50
xylose-no β-ala	.003	0.03	0.150	–0.28
arabinose	.054	0.02[c]	0.643	–0.73[c]
glucose	.0075	–0.01[c]	0.304	–0.57[d]
galactose	.014	0.01[c]	0.790	–0.26
galactose-no β-ala	.001		0.041	
mannose	.0074	0.01[c]	0.021	–0.03
rhamnose	.0020	–0.09[c]	0.094	–0.16[c]
fructose	.033	0.02[c]	0.367	–0.18
lactose	.031	–0.04[c]	0.213	

[a] Initial concentrations: sugars 0.10 M, β-alanine 0.033 M [b] Changes in A_{420} and pH after 80 min reaction at ca. 100 °C. [c] Change after 160 min. [d] Change after 300 min.

Reaction pH initially at 7.3 ± 0.04 decreased during most reactions, indicating the formation of strong acid(s) (**Table 1**). However, the fact that large A_{420} changes were consistently induced by phosphate at nearly constant pH (cf. xylose, galactose, and fructose reactions, **Table 1**) suggested that phosphate was acting independently of hydrogen and/or hydroxide ion concentration to influence browning.

Various reducing sugars differed in their propensity for browning in contact with phosphate with or without β-alanine present (**Table 1**). The effect of phosphate was estimated by comparing the browning produced in phosphate buffer with the browning obtained in similar reactions using a relatively unreactive, bis−tris buffer (pK_a 6.5). All sugars showed enhanced Maillard browning in phosphate buffer versus bis−tris buffer. In general, pentoses (ribose, xylose and arabinose) displayed greater browning than hexoses (glucose, mannose and rhamnose). Among the hexoses, galactose and fructose showed relatively high browning potential, comparable with the pentoses. Browning tendencies among individual sugars are not clearly understood, but are probably related to differing amounts of acyclic (carbonyl-form) isomers available at equilibrium in buffers at 100 °C (*7*).

The possibility that phosphate-induced browning was caused by the presence of trace transition metal ion impurities was eliminated by two control experiments. In one, EDTA (ethylenediaminetetraacetic acid) was included at 0.0033 M in the ribose/β-alanine/phosphate reaction described in **Table 1**, resulting in 88% of the A_{420} found without added EDTA. Thus, EDTA, a strong complexer of metal ions, failed to significantly reduce the browning effect of phosphate. In the second experiment, copper(II) sulfate was included at 50 μM in the ribose/bis−tris reaction. No change in A_{420} was observed within our error limit, thereby reducing the probability of browning being due to metal ion catalyzed sugar degradation.

A 0.10 M bisulfite/sulfite buffer led to less browning in the xylose/β-alanine system compared to 0.10 M phosphate. Under **Table 1** conditions (80 min reaction, initial pH 7.24), an A_{420} of 0.191 (Δ pH = + 0.18) was obtained compared to 0.837 with phosphate. The chemical reducing property of bisulfite may have contributed to the reduced browning effect, but this possibility remains to be established.

Enhanced browning was also observed in carboxylate-based buffers. The A_{420} produced by ribose/β-alanine in 0.10 M acetate buffer under **Table 1** conditions (0.15 after 80 min/100 °C) was significantly less than 1.17 seen in phosphate; however, it was

still greater than 0.061 obtained in inert bis/tris buffer. The pK_a of acetic acid (4.76) was too low to enable effective buffer action near neutral pH, and a relatively large pH change during the reaction (−1.33) may have influenced color formation in acetate. Dibasic organic acids with pK_2 closer to the pK_2 of phosphoric acid (7.12) were better buffers near pH 7 and also produced enhanced browning similar to phosphate. For example a xylose/β-alanine reaction in succinate buffer (pK_2 5.6) at an initial pH of 7.14 developed an A_{420} of 0.249 in 80 min, with a pH change of − 0.78. For comparison, a similar reaction run in bis−tris buffer led to an A_{420} of only 0.050 (**Table 1**). Under identical conditions using 0.1 M citrate buffer at an initial pH of 7.33, the xylose/β-alanine reaction developed an A_{420} of 0.379 (Δ pH = − 0.87). The effect of citrate on browning in a glucose−glycine model system was observed to diminish relative to phosphate under milder reaction conditions (pH 7, 25 °C) (*5*).

Whereas this study is concerned mainly with Maillard browning and nonvolatile products formed near neutral pH, a recent report by Mottram and Norbrega described the accelerating effects of phosphate and phthalate ions on the formation of flavor volatiles in a cysteine/ribose system at acidic pH (*8*). The catalytic activity of phosphate at acid pH suggested the involvement of dihydrogen phosphate ion in the Maillard reaction mechanism.

Sugar Degradation by Buffer Anions. The effect of phosphate ion on sugars alone is also illustrated in **Table 1**. The three sugars that produced the greatest Maillard browning (ribose > xylose > galactose) also browned to a lesser degree but in the same order of reactivity in the absence of β-alanine. Also, as found in the Maillard reactions, browning was virtually eliminated in the total absence of phosphate ion.

The degree of ribose browning decreased markedly with lower phosphate concentration or in the case of a phosphate monoester. Under **Table 1** conditions, reacting 0.1 M ribose for 80 min, 0.05 M phosphate in 0.10 M bis−tris buffer (initial pH 7.3) developed an A_{420} of only 0.045 compared to 0.262 obtained in 0.10 M phosphate. Also, in a similar reaction, a phosphate monoester, DL-α-glycerophosphate at 0.05 M in 0.10 M bis−tris buffer (initial pH 7.3) gave a lower but comparable browning effect: A_{420} 0.026. A control experiment in 0.1 M bis−tris buffer produced no visible browning under the same conditions.

To gain insight into the effects of buffer anions on sugars alone, we investigated reaction products for the presence of 1,2-dicarbonyl fragments, which are known to be key intermediates for color formation in the Maillard reaction. The ribose/phosphate reaction was chosen as a model for study, and (OPD) was used to trap 1,2-dicarbonyls as their more easily analyzed quinoxaline derivatives. The ribose/phosphate reaction mixture described in **Table 1** was treated with OPD and analyzed qualitatively by GC/MS. Four volatile quinoxalines were identified, namely quinoxaline, 2-methyl, 2,3-dimethyl, and 2-ethylquinoxaline, corresponding to the dicarbonyls shown in **Figures 2** and **3**; ethanedial (glyoxal) **1**, 2-oxopropanal (pyruvaldehyde) **2**, 2,3-butanedione (diacetyl) **3,** and 2-oxobutanal **4**. On the basis of quinoxaline peak areas, the relative proportions of **1**, **2**, **3**, and **4** were 0.29, 1.00, 0.035, and 0.063, respectively. Also, a control reaction using bis−tris buffer instead of phosphate gave no detectable quinoxalines after OPD treatment. Clearly, phosphate ion accelerated the fragmentation of ribose into 1,2-dicarbonyls at neutral pH, where the effect of hydrogen and/or hydroxide ion appeared to be negligible.

The mode of ribose fragmentation was further investigated by TLC analysis of the less volatile quinoxaline products from

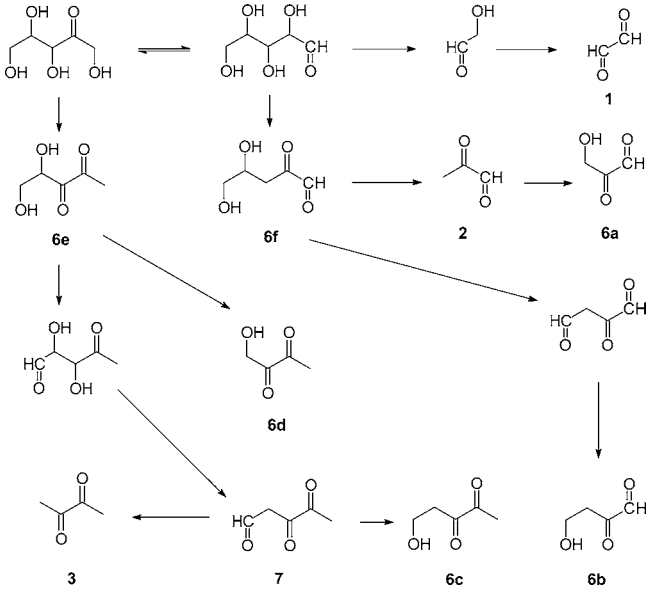


Figure 2. Formation of 1,2-dicarbonyl compounds in ribose degradation catalyzed by phosphate ion.

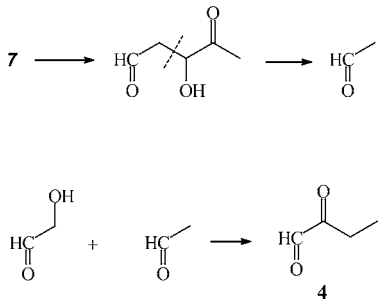
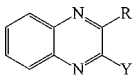


Figure 3. Formation of 2-oxobutanal, compound 4.

the ribose/phosphate reaction. Silica gel TLC indicated a major component in the R_f region previously known to contain quinoxaline derivatives of 5-carbon deoxyosones (*9*). A control experiment without phosphate produced none of this material. Preparative TLC in this R_f region provided milligram quantities of a substance whose UV spectrum (237 nm log ϵ 4.35, 317 nm log ϵ 3.78) was consistent for a quinoxaline structure or mixture of quinoxalines of MW ca. 200 (*10*). Typically, 0.9 g of ribose afforded ca. 40 mg of quinoxalines after prep TLC (3.3% molar yield from ribose). Analysis of the substance by 300 MHz proton NMR provided tentative evidence for the quinoxaline mixture **5a**−**5e**, (**Table 2**) based on comparisons with published NMR data (*9*−*11*). From peak area measurements, the relative amounts present were: **5e** > **5c** ∼ **5d** ≫ **5a** ∼ **5b**. Approximately 70% of the mixture consisted of 2-methylquinoxaline derivatives based on peak area ratios. Analysis of the mixture by reversed phase TLC (RP-18) separated four components at R_f values 0.67 (trace), 0.73 (trace), 0.78 (major), and 0.86 (minor) in general agreement with NMR data. Quinoxalines **5a**−**5e** are derived from a corresponding group of 1,2-dicarbonyl compounds, **6a**−**6e** arising from ribose degradation, namely 3-hydroxy-2-oxopropanal, **6a**; 4-hydroxy-2-oxobutanal, **6b**; 5-hydroxy-2,3-pentanedione, **6c**; 4-hydroxy-2,3-butanedione, **6d**; and 4,5-dihydroxy-2,3-pentanedione, **6e**. Minor peaks in the quinoxaline mixture spectrum suggested that a trace of 2-oxo-4,5-dihydroxypentanal (3-deoxyribosone) **6f** may also have been present in the original reaction mixture.

A scheme depicting ribose degradation in terms of the major 1,2-dicarbonyl products observed is shown in **Figure 2**. Many

Table 2. Proton NMR Data for 2-R-3-Y-substituted Quinoxalines Derived from Phosphate Ion Catalyzed Degradation of Ribose[a]



5a - 5e

		Y		
compd	*R*	CH_2OH	CH_2CH_2OH	$CH(OH)CH_2OH$
5a	H, 8.74(s)	5.28(s)		
5b	H, 8.72(s)		3.24(t) [5.4] 4.16(t) [5.7]	
5c	CH_3, 2.70(s)		3.06(t) [5.4] 4.22(t) [5.4]	
5d	CH_3, 2.79(s)	4.91(s)		
5e	CH_3, 2.79(s)			3.85(dd) [11.7, 5.4] 4.03(dd) [11.5, 3.6] 5.11(dd) [5.5, 3.3]

[a] Chemical shift data are in ppm; (multiplicity) s = singlet, t = triplet, and dd = doublet of doublets; [J in Hz]. Compounds **5a**–**5e** each exhibited complex multiplets at 7.70 and 7.99 ppm for benzene ring protons.

of the reactions described are isomerizations, dehydrations and aldol/retroaldol processes that are already well documented in the literature of carbohydrate chemistry (*12*). Key to the formation of all products is the equilibration (isomerization) of aldose ↔ ketose sugars. In the case of ribose, it is ribose ↔ ribulose. Sugar dehydration leads to the formation of deoxyosones, **6e** and **6f**. Retroaldol reaction (RA) of 3-deoxyosone **6f** explains the formation of pyruvaldehyde, **2**. The 1-deoxyosone **6e** may be the progenitor of additional dicarbonyls **3**, **4**, and **6d**. RA of **6e** with loss of formaldehyde could generate **6d** directly, whereas **3** and **4** require more circuitous routes. Isomerization of **6e** followed by dehydration can afford 3,4-dioxopentanal **7**, which by hydrolysis and loss of formic acid could produce **3**. The drop in pH during the ribose/phosphate reaction is consistent with formic acid formation and provides additional indirect evidence for **7**.

Compounds **1**, **4**, **6a**, **6b**, and **6c** are chemically unique in that oxidation/reduction steps appear to be needed for their formation. Conceivably, hydroxyacetaldehyde from RA of ribose is oxidized (− 2H) to yield **1**. The unusual dicarbonyl **4** is probably formed by the aldol condensation/dehydration of hydroxyacetaldehyde originating from ribose (**Figure 2**) and acetaldehyde (**Figure 3**). In addition, the acetaldehyde required for this reaction could derive from **7,** following C-3 carbonyl reduction (+ 2H) and RA. A C-1 carbonyl reduction of **7** might also explain the origin of **6c**. Minor product **6a** may be derived via oxidation of **2** (+ O) or from **6f** after oxidation at C-3 (+ O) followed by RA reaction. Compound **6b** is conceivably derived from the 3-deoxyosone **6f**. Oxidation at C-3 of **6f** (+ O) (i.e., formation of ribosone) followed by fragmentation with loss of water and HCHO would afford a four carbon tricarbonyl (2,4-dioxobutanal) which upon reduction at C-4 could yield **6b**. Redox processes appear to be an integral part of Maillard chemistry; however, their mode of action remains a mystery and is therefore a subject worthy of future research.

Surprisingly, no definitive evidence was found for the relevant 3-deoxyosone **6f** except for its possible degradation products **2, 6a, and 6b**.

Mechanism of Catalytic Activity. The apparent enhanced basicity of polyatomic anions compared with hydroxyl ion is not obvious, because electron delocalization in the polyatomic anions should make them very weak bases and therefore much less effective for proton withdrawal than hydroxyl ions.

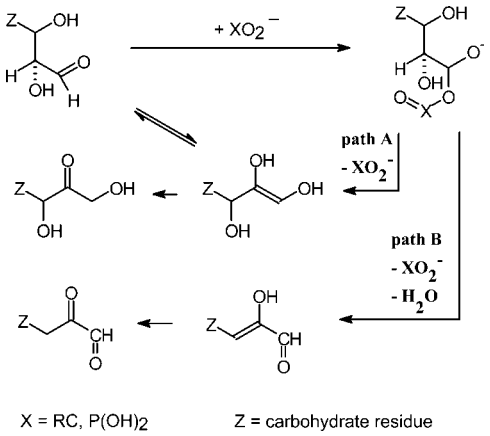


Figure 4. Reactions of aldose sugars with polyatomic anions.

Apart from a possible concentration effect due to high buffer strength compared to hydroxyl ion concentration near neutral pH the catalytic activity of carboxylate and dihydrogenphosphate ions can be explained by an intramolecular effect involving their common structural element: $O = X - O^- \leftrightarrow {}^-O - X = O$ (abbreviated XO_2^-) where X = RC for carboxylate and X = $P(OH)_2$ for dihydrogenphosphate (**Figure 4**). We suggest that catalytic activity begins with nucleophilic addition of XO_2^- to the sugar carbonyl. For phosphate ions, nucleophilic addition is well documented in catalysis of hydrolysis reactions (*13*). Addition of XO_2^- to a reducing sugar carbonyl group orients the X = O function in a favorable position to abstract a proton from the α-carbon atom of the sugar leading either to enolization (path A) or dehydration (path B). Path A facilitates aldose ↔ ketose isomerization, whereas path B results in the formation of a 3-deoxyosone (e.g., **6f** in the case of ribose). An intrinsic catalytic effect of XO_2^- can therefore be attributed to the possibility of a more efficient (intramolecular) proton abstraction process compared to the intermolecular acid−base reaction otherwise available for anions including hydroxide ion. The same mechanism should apply for a wide variety of carboxylates, substituted phosphates, and related arsenates. Actual intermediates involved in proton transfer are a matter for further speculation. At pH 7, phosphoric acid and carboxylic acids can probably be excluded because of their low p*K* values. A possible intermediate might be a zwitterionic species (i.e., $^-O - X^+ -$ OH), which later transfers a proton to enolate or hydroxide ions to regenerate the catalyst.

In theory, monohydrogen phosphate ion, HPO_4^{-2} might also act as a catalyst by following a similar mechanism. Our data does not exclude this possibility; however, we favor the XO_2^- hypothesis because it uniquely includes the catalytic behavior of carboxylate ions.

In conclusion, our experiments suggest that polyatomic anions of the form XO_2^- probably facilitate the aldose/ketose equilibration of reducing sugars and aldose sugar dehydration to form 3-deoxyosones. Subsequent reactions apparently lead to additional α-dicarbonyl formation. Also, because α-dicarbonyls are key intermediates that lead to colored melanoidins, the extra α-dicarbonyls arising from XO_2^--catalyzed sugar degradation explains the enhanced browning observed overall in XO_2^--catalyzed Maillard reactions.

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