Degradation of L-Ascorbic Acid and Mechanism of Nonenzymic Browning Reaction

Part II.* Non-oxidative Degradation of L-Ascorbic Acid Including the Formation of 3-Deoxy-L-pentosone

By Tadao Kurata** and Yosito Sakurai

Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo

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L-Ascorbic acid, in an acid condition, was degraded to furfural with the formation of 3-deoxy-L-pentosone as an intermediate. Furfural and 3-deoxy-L-pentosone were isolated and identified as 2,4-dinitrophenylhydrazone and bis-2,4-dinitrophenylhydrazone, respectively.

This acid-catalyzed degradation reaction took place without oxygen and under the storage or cooking condition of foodstuffs. It was shown that aldopentoses and 2-keto-L-gulonic acid themselves were not intermediates of the reaction. D-Araboascorbic acid was also degraded in the same way to furfural and 3-deoxy-D-pentosone.

A mechanism for the non-oxidative degradation reaction of L-ascorbic acid, including the formation of 3-deoxy-L-pentosone and furfural, was proposed.

Degradation of L-ascorbic acid can be classified into two types of reactions, namely the non-oxidative and the oxidative. One of the characteristic differences between these two reactions is that furfural is much more easily produced through the former.

Since the report by Hirst et al.,¹ the formation of furfural from L-ascorbic acid (ASA) in strong acid media has been confirmed by many workers and some reaction mechanisms for the formation of furfural from ASA have been proposed.²,³ However, none of them seems to be acceptable as the mechanism which takes place in ordinary foodstuffs.

The object of this paper is to elucidate the reaction mechanism for the formation of furfural in non-oxidative degradation of ASA taking place in the nonenzymic browning reaction of foodstuffs of high ASA content, especially of concentrated lemon or orange juice, under the storage (keeping the sample at 38°C for 24 days) or the cooking condition (heating the sample in a boiling water bath for an hour).

Degradation of ASA under the Cooking Condition

Recent developments in the study of the reaction mechanism of the degradation of L-ascorbic acid include the formation of furfural and 3-deoxy-L-pentosone. However, the mechanisms proposed by various workers do not seem to be acceptable for ordinary foodstuffs.

Degradation of ASA in strong acid media has been confirmed by many workers. The formation of furfural from ASA in strong acid media has been shown to take place without oxygen and under the storage or cooking condition of foodstuffs. Aldopentoses and 2-keto-L-gulonic acid themselves were not intermediates of the reaction. D-Araboascorbic acid was also degraded in the same way to furfural and 3-deoxy-D-pentosone.

A mechanism for the non-oxidative degradation reaction of L-ascorbic acid, including the formation of 3-deoxy-L-pentosone and furfural, was proposed.

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Degradation of L-Ascorbic Acid and Mechanism of Nonenzymic Browning Reaction. Part II 171

sugars and the sugar-amino reaction, revealed the formation of 3-deoxyglycosones as an intermediate to 2-furaldehydes. The assumption that partly the same kind of reaction may take place in the degradation of ASA was substantiated by showing the formation of 3-deoxy-L-pentosone as an intermediate to furfural, as follows.

ASA in buffer solution (pH 2.2, 0.5 M) was degraded under the cooking condition, and the resulting slightly yellow colored solution was treated with the 2,4-dinitrophenylhydrazine (2,4-DNPH) reagent. 2,4-Dinitrophenylhydrazone (2,4-DNP) mixture obtained was submitted to thin layer chromatography (TLC), and the resulted thin layer chromatogram (TLCm) showed two main spots other than 5~8 weak spots. Both 2,4-DNPs corresponding to the two main spots were isolated in crystalline form by column chromatography of acid alumina, and identified as furfural 2,4-DNP and 3-deoxy-L-pentosone (3-D-P) bis-2,4-DNP by their melting points, elementary analysis, infrared spectra and ultraviolet spectra.

Almost the same results as mentioned above were obtained by degrading ASA in 5% sulfuric acid solution, and also by degrading 2-keto-L-gulonic acid (KGA) in the buffer solution (pH 2.2).

The fact that the amounts of furfural and 3-D-P yielded by the degradation in 5% sulfuric acid solution were much higher than those obtained in the buffer solution (pH 2.2) indicates that these degradation reactions are accelerated by acid catalyst. The dehydration reaction of 3-D-P to furfural is also accelerated by acid catalyst, because the differences of the yield between furfural and 3-D-P were much larger in the degradation in 5% sulfuric acid than in the degradation at pH 2.2.

Although clear separation of L-xylosone bis-

2,4-DNP and 2,3-diketo-L-gulonic acid (DKG) bis-2,4-DNP was rather difficult by silica gel column chromatography, their formation in the degradation at pH 2.2 was positively confirmed by TLC.

Roughly speaking, the sum of 3-D-P and furfural represents the amounts of carbonyl compounds produced through the non-oxidative degradation, and the sum of L-xylosone and DKG represents those through the oxidative one. Therefore, under the cooking condition, the main degradation path of ASA apparently is non-oxidative.

The fact that KGA which has been considered to be an intermediate in the non-oxidative degradation of ASA in strongly acid media produced less furfural and 3-D-P than ASA in the buffer solution of pH 2.2, indicates that KGA itself is not an intermediate in the reaction.

D-Araboascorbic acid is also degraded to furfural and 3-deoxy-D-pentosone under the cooking condition at pH 2.2.

ASA and D-araboascorbic acid (D-ASA) are more easily degraded to furfural than D-ribose. Since aldopentoses do not degrade to furfural at pH 2.2, aldopentoses are not intermediates in the reaction.

Degradation of ASA under the Storage Condition

Under the storage condition, ASA yielded essentially the same degradation products as those obtained under the cooking condition. In this case, L-xylosone and DKG were obtained in higher yields in the presence of oxygen than in the absence of it. The yields of 3-D-P and furfural, however, were unchanged, which indicates that furfural formation reaction is not affected by the presence of oxygen. As the acid catalyzed dehydration reaction (3-D-P→furftural) did not take place so rapidly under the storage condition as under the cooking condition, some accumulations of 3-D-P


* Strictly speaking, the yields of 2,4-DNP described above do not represent the correct amount of carbonyl compounds, because each carbonyl compound has a different reactivity to 2,4-DNPH reagent.
FIG. 1. The Non-oxidative Degradation of L-Ascorbic Acid.
were observed (see also Table I). It was also
demonstrated that a considerable amount of
ASA was degraded through the non-oxidative
degradation even under the oxidative storage
condition. Since n-xylose, an aldopentose, is
rather stable under the storage condition, it
cannot be an intermediate of the furfural
formation from ASA.

Concerning the discoloration of concentrated
sweetened fruits juice, it has been confirmed
that the main substance which causes dis-
coloration is D-fructose originated from added
sucrose, producing 3-deoxy-D-glucosone and 5-
(hydroxymethyl)-2-furfural as intermediates.3
However, in the case of fruits juice5 or even
in concentrated sweetened juice when ASA
content is high, ASA is also responsible for
browning.

**Mechanism of the Non-oxidative Degradation
of ASA**

On the basis of these data, it seems reason-
able to assume that the first step of the non-
oxidative degradation of ASA in an acid
condition is hydrolysis of lactone ring followed
by decarboxylation and dehydrations forming
3-D-P and furfural. Although there is no
conclusive evidence, at present, to determine
which one of the two reactions, the dehydra-
tion and the decarboxylation, takes place first
after the cleavage of lactone ring, it seems
more probable that the dehydration precedes
the decarboxylation. The mechanism proposed
for the non-oxidative degradation of ASA,
including the two possible types of path, is
given in Fig. 1.

**EXPERIMENTAL**

Melting points were uncorrected. For absorption
measurements in visible and ultraviolet regions, Cary
Model 14 Auto-recording Spectrophotometer was used.

**Chromatography**

a) Acid alumina column chromatography.

Column chromatography used here was virtually
identical with the method described by Kato,8 except
that alumina (Merck) was suspended in 1 N hydrochloric
acid and allowed to stand overnight.

b) Silica gel column chromatography. Silica gel
(Mallinckrodt, for chromatography; or Kanto Chemical,
for chromatography, 100 mesh) was suspended in toluene
and packed in a column. The 2,4-DNP mixture was
dissolved in ethyl acetate or other inactive and volatile
solvent. The resulted 2,4-DNP solution was adsorbed
to a small amount of silica gel with constant mixing.
After removal of the solvent at room temperature,
the silica gel was suspended in the solvent used for
packing of the column or the first eluant. The ob-
tained silica gel suspension was poured on the column
to form the adsorbed layer of the sample. Elution
was carried out in the usual manner.

The length of the column required depends on the
number of the components of the sample and the
solvent system used for elution. For ordinary use,
the length of the column is preferably 14~15 times
longer than that of adsorbed layer of the sample.
For the semiquantitative purpose, the length of the
adsorbent layer is about 30 cm and the adsorbed layer
of the sample is 1.5~1.8 cm. The solvent system for
elution was previously examined by thin layer chro-
matography.

c) Thin layer chromatography.9 Silica Gel G
was used as adsorbent. Toluene or toluene-ethyl
acetate mixture (3 : 1, 1 : 1) was used for ordinary
developers.

**Preparation of the 2,4-DNP mixture for silica gel
column chromatography.** After the degradation
reaction was over, the reaction mixture was cooled,
treated with the 2,4-DNPH reagent.10 The 2,4-DNP
precipitated within 30 minutes was collected by filtra-
tion, and dissolved in ethyl acetate. The ethyl acetate
solution was washed with 2 N hydrochloric acid, then
plenty of water, and dried over anhydrous sodium
sulfate, concentrated under reduced pressure, and the
residue was dried over silica gel in vacuum.

**Deoxygenation of the buffer mixture for the
storage experiments.** Deionized water was used in
the storage experiments. Clark-Lubs' buffer mixture,
after boiling for 20 minutes, was cooled to room

10) R. L. Shriner and R. C. Fuson, "The Systematic
Identification of Organic Compounds", John Wiley
& Sons, 1948, p. 171.
temperature under constant bubbling with purified nitrogen gas. ASA or other samples were dissolved in this buffer solution and kept in flasks with head spaces replaced by purified nitrogen gas, tightly stoppered and sealed with paraffin.

**Isolation and identification of furfural 2,4-DNP and 3-D-P bis-2,4-DNP produced from ASA under the cooking condition**

ASA (17.6 g) was dissolved in 200 ml of Clark-Lubs' (KCl-HCl) buffer solution (pH 2.2) and heated in a boiling water bath for an hour, cooled and treated with the 2,4-DNPH reagent (4 g of 2,4-dinitrophenylhydrazine). After standing for 30 minutes, the resulting precipitate was filtered and dissolved in ethyl acetate. This ethyl acetate solution was washed with 2 N hydrochloric acid, water and dried over anhydrous sodium sulfate, concentrated under reduced pressure to a small volume and charged on a column of acid alumina packed with ethyl acetate. The column was first eluted with ethyl acetate. The main component of the fraction eluted with ethyl acetate was furfural 2,4-DNP, obtained as red prisms (anti-form) after recrystallization three times from toluene (m.p. 225°C). The ultraviolet spectrum ($\lambda_{max}$ 388 mp), the infrared spectrum and behavior on TLCm were identical with those of authentic sample.

The column was then eluted with ethyl acetate-ethanol mixture (25 : 1 to 19 : 1) with increasing ethanol concentration, and the fractions mainly contained 3-D-P bis-2,4-DNP were combined, evaporated under reduced pressure, and crude 3-D-P bis-2,4-DNP was obtained in crystalline powder. This crude 3-D-P bis-2,4-DNP containing some impurities was rechromatographed, and pure 3-D-P bis-2,4-DNP recrystallized from ethyl acetate-ethanol mixture was obtained as orange-red fine prisms (m.p. 259°C, dec.). Recrystallization from acetone-ethyl acetate mixture yielded needles (m.p. 259°C, dec.). *Anal. Found: C, 37.61; H, 3.42; N, 23.01%. Calcd. for C$_{17}$H$_{16}$N$_{8}$O$_{10}$: C, 37.10; H, 3.28%. $\lambda_{max}$: 434 mp; [\alpha]$_{D}$~$^{29}$~304° ($\epsilon$ 0.73 in dioxane); the behavior on TLCm was identical with that of authentic sample. The infrared spectrum was identical with that of the crystal B of authentic 3-D-P bis-2,4-DNP* as shown in Fig. 2.

Furfural 2,4-DNP (m.p. 225°C) and 3-D-P bis-2,4-DNP (m.p. 259°C) were also obtained from the degradation solution of ASA under the cooking condition

ASA (8.8 g) was dissolved in 5% sulfuric acid and heated for an hour in a boiling water bath. After cooling, the reaction mixture was treated with the 2,4-DNPH reagent, and 2,4-DNP precipitated within 30 minutes was collected. The yield of 2,4-DNP mixture obtained was 2,066 mg, and 250 mg of it was chromatographed over silica gel.

Both ASA (8.8 g) and KGA (9.7 g) were also degraded in pH 2.2 buffer solution under the same experimental condition, and 263 mg and 90 mg of 2,4-DNP mixture were obtained from ASA and KGA, respectively. Both 2,4-DNP mixtures were chromatographed over silica gel.

Some procedures for column chromatography of silica gel were already described. The column was first eluted with toluene. After furfural 2,4-DNP was eluted, the column was eluted with the mixture of toluene-ethyl acetate (6:1 to 3:1) with increasing ethyl acetate concentration, and 3-D-P bis-2,4-DNP

* Offered by Dr. H. Kato.

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**Preparation of KGA and its degradation in pH 2.2 solution**

KGA was prepared from diacetone-2-keto-L-gulonic acid by the method similar to that described by Reichstein and Grüssner.\(^{11}\) KGA was obtained as colorless fine needles (m.p. 171~172°C). *Anal. Found: C, 37.50; H, 5.28%. Calcd. for C$_{6}$H$_{10}$O$_{7}$: C, 37.10; H, 5.19%.

Furfural 2,4-DNP (m.p. 225°C) and 3-D-P bis-2,4-DNP (m.p. 259°C) were also obtained from the degradation solution (pH 2.2) of KGA heated in a boiling water bath for an hour.

**Yields of some carbonyl compounds produced from ASA under the cooking condition**

ASA (8.8 g) was dissolved in 5% sulfuric acid and heated for an hour in a boiling water bath. After cooling, the reaction mixture was treated with the 2,4-DNPH reagent, and 2,4-DNP precipitated within 30 minutes was collected. The yield of 2,4-DNP mixture obtained was 2,066 mg, and 250 mg of it was chromatographed over silica gel.

Both ASA (8.8 g) and KGA (9.7 g) were also degraded in pH 2.2 buffer solution under the same experimental condition, and 263 mg and 90 mg of 2,4-DNP mixture were obtained from ASA and KGA, respectively. Both 2,4-DNP mixtures were chromatographed over silica gel.

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L-xylosone bis-2,4-DNP and DKG bis-2,4-DNP were successively eluted in this order with considerable overlapping of the latter two. Each fraction was examined its purity by TLC and rechromatographed, if necessary. The fractions containing almost one component were combined and evaporated under reduced pressure, dried, and weighed. The results are given in Table I. Since clear separation of DKG bis-2,4-DNP and L-xylosone bis-2,4-DNP was rather difficult, the sum of both 2,4-DNPs was chosen for comparison of yields. Main component of this fraction was DKG bis-2,4-DNP and L-xylosone bis-2,4-DNP was contained in very small amount. From the reaction solution of ASA degraded in 5% sulfuric acid, neither DKG bis-2,4-DNP nor L-xylosone bis-2,4-DNP was obtained.

This silica gel column chromatography is not adequate for a strict quantitative analysis, for each fraction contains some impurities, however, is useful enough for comparison of relative amount of each fraction.

**Degradation of aldopentoses in the buffer solution**

D-Xylose (7.5 g) was dissolved in 100 ml of Clark-Lubs’ buffer (pH 2.2) solution and heated in a boiling water bath for an hour. On treating with the 2,4-DNPH reagent, very small amount of 2,4-DNP was formed. The 2,4-DNP mixture obtained was submitted to TLC, and only trace amount of furfural 2,4-DNP and 3-D-P bis-2,4-DNP were detected.

Each 3 mM of L-xylose, D-arabinose, D-ribose, ASA and D-ASA was dissolved respectively in about 6 ml of Clark-Lubs’ buffer (pH 2.2) solution, and heated in a boiling water bath for an hour. On treating with the 2,4-DNPH reagent, both reaction solutions of ASA and p-ASA produced considerable turbidity within 5 minutes, and then precipitated 2,4-DNP mixture which consisted mainly of furfural 2,4-DNP and 3-D-P bis-2,4-DNP was examined by TLC. On the other hand, the reaction solutions of three aldopentoses treated in the same way, produced no turbidity and precipitated no 2,4-DNP within 5 minutes, and formed only crystals of 2,4-dinitrophenylhydrazine after standing over 10 minutes.

**Degradation of d-ASA in the buffer solution**

D-ASA (5 g) was dissolved in about 57 ml of Clark-Lubs’ buffer solution (pH 2.2) and heated for an hour in a boiling water bath. The 2,4-DNP mixture obtained was fractionated with silica gel column chromatography. The column was first eluted with toluene, and then toluene-ethyl acetate mixture (6:1→3:1), with increasing concentration of ethyl acetate. Furfural 2,4-DNP was obtained as red prisms, m.p. 225°C (yield, 40 mg). 3-D-P bis-2,4-DNP was obtained as orange-red fine prisms, m.p. 259°C. Anal. Found: C, 41.41; H, 3.61; N, 22.70%. Calcd. for C17H16O8N10: C, 41.47; H, 3.28; N, 22.76%. The infrared spectrum was identical with that of crystal B of authentic 3-D-P bis-2,4-DNP: v_max 434 cm⁻¹; [α]_D^294° (c 0.16, in dioxane); the behavior on TLC was similar to that of authentic sample.

**Carbonyl compounds produced from ASA under the storage condition**

ASA (17.6 g) was dissolved in 2 l of Clark-Lubs’ buffer solution (pH 2.3). This 0.05 M ASA solution was kept at 38°C in loosely corked Erlenmeyer flask (2 l). (sample-A) Another 2 l of 0.05 M ASA solution prepared by using deoxygenated buffer mixture was kept in a flask tightly stoppered and sealed with paraffin. (sample-B) After 24 days’ storage both stock solutions were cooled at room temperature and the discoloration was examined by visual assessment. Sample-A was considerably colored in yellowish-brown but the discoloration of sample-B was almost negligible. The 2,4-DNPH reagent (4 g of 2,4-DNPH) was

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**Table I. Formation of Carbonyl Compounds under the Cooking Condition**

<table>
<thead>
<tr>
<th>Materials (g)</th>
<th>Solvents</th>
<th>Total* 2,4-DNP (mg)</th>
<th>Furfural** 2,4-DNP (mg)</th>
<th>3-D-P bis** 2,4-DNP (mg)</th>
<th>L-Xylosone bis-2,4-DNP+DKG bis-2,4-DNP (mg)</th>
<th>Browning: visual assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA 8.8</td>
<td>5% H2SO4</td>
<td>2,066</td>
<td>1,352 [470]</td>
<td>463 [124]</td>
<td>+++ brown</td>
<td>+</td>
</tr>
<tr>
<td>ASA 8.8</td>
<td>KCl-HCl buf. (pH 2.2)</td>
<td>263</td>
<td>85 [30]</td>
<td>87 [23]</td>
<td>29 + straw</td>
<td>-</td>
</tr>
<tr>
<td>KGA 9.7</td>
<td>KCl-HCl buf. (pH 2.2)</td>
<td>90</td>
<td>27 [9]</td>
<td>28 [8]</td>
<td>6 -</td>
<td>-</td>
</tr>
</tbody>
</table>

* Amounts of 2,4-DNP precipitated within 30 min.
** Values in brackets indicate the amounts of original carbonyl compounds (Calcd. from the yields of 2,4-DNPs).
*** No 2,4-DNP was obtained.
Formation of some carbonyl compounds from ASA, D-fructose and D-xylose under the storage condition

ASA (1.76 g), D-fructose (1.8 g) and D-xylose (1.5 g) were dissolved respectively in 200 ml of Clark-Lubs' buffer solution which was deoxygenated before use, and the solutions were stored in brown color Erlenmeyer flask (200 ml) tightly stoppered and sealed with paraffin.

After 24 days' storage at 38°C, each stock solution was examined its discoloration by visual assessment, pH values with a pH meter. Fifteen ml of the 2,4-DNPH reagent was added to each stock solution and 2,4-DNP precipitated within 30 minutes, in case of ASA, was collected and submitted to TLC; the main components were found to be furfural and 3-D-P, but no precipitates were observed within 30 minutes in case of aldoses. Stock solution of ASA was slightly colored but those of aldoses were completely colorless. No changes in pH values were observed in case of ASA, and slightly higher values were obtained in case of aldoses. Ultraviolet spectra revealed the formation of 2-furaldehydes in case of aldoses, but the yields were low. Results are shown in Table III.

<table>
<thead>
<tr>
<th>Materials* (g)</th>
<th>Total** 2,4-DNP (mg)</th>
<th>Furfural*** 2,4-DNP (mg)</th>
<th>3-D-P**** bis-2,4-DNP (mg)</th>
<th>L-Xylosone bis-2,4-DNP + DKG (mg)</th>
<th>Browning: visual assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA 1.76</td>
<td>107</td>
<td>+</td>
<td>+</td>
<td>+****</td>
<td>2.2</td>
</tr>
<tr>
<td>D-Fru. 1.8</td>
<td>0~trace</td>
<td>*****</td>
<td></td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>D-Xyl. 1.5</td>
<td>0~trace</td>
<td>-****</td>
<td></td>
<td></td>
<td>2.2</td>
</tr>
</tbody>
</table>

* In KCl-HCl buf. (pH 2.2) soln. without oxygen.
** Amounts of 2,4-DNP precipitated within 30 min. after treatment with 2,4-DNPH reagent.
*** Confirmed by TLC.
**** No discoloration was observed with 1 cm solution layer.
***** Absorption of 5-(hydroxymethyl)-2-furfural was observed in UV spectrum.
****** Absorption (shoulder) of furfural was observed in UV spectrum.

Tab. II. FORMATION OF CARBONYL COMPOUNDS UNDER THE STORAGE CONDITION

<table>
<thead>
<tr>
<th>Materials* (g)</th>
<th>Oxygen</th>
<th>Total** 2,4-DNP (mg)</th>
<th>Furfural*** 2,4-DNP (mg)</th>
<th>3-D-P**** bis-2,4-DNP (mg)</th>
<th>L-Xylosone bis-2,4-DNP + DKG (mg)</th>
<th>Browning: visual assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA 17.6</td>
<td>+</td>
<td>1,992</td>
<td>310 [108]</td>
<td>620 [160]</td>
<td>&gt; 680</td>
<td>+</td>
</tr>
<tr>
<td>ASA 17.6</td>
<td>-</td>
<td>1,410</td>
<td>302 [105]</td>
<td>638 [171]</td>
<td>308</td>
<td>-****</td>
</tr>
</tbody>
</table>

* 0.05 M, in KCl-HCl buf. (pH 2.3) soln.
** Amounts of 2,4-DNP precipitated within 30 min. after treatment with 2,4-DNPH reagent.
*** Values in brackets indicate the amounts of original carbonyl compounds. (Calcd. from the yield of 2,4-DNPs)
**** Discoloration was positively observed with a thick solution layer.

Tab. III. FORMATION OF CARBONYL COMPOUNDS FROM ASA, D-FRUCTOSE AND D-XYLOSE UNDER THE STORAGE CONDITION

Added to each of the sample solution, and the 2,4-DNP precipitated within 30 minutes was collected and dissolved in ethyl acetate. Ethyl acetate solution was washed with 2 N hydrochloric acid, water, and dried over anhydrous sodium sulfate, then concentrated to a minimum volume and dried over silica gel in vacuum. The yields of the 2,4-DNP mixture obtained from sample-A and -B were 1,992 mg and 1,410 mg, respectively. Two hundred and fifty mg of each 2,4-DNP mixture from sample-A and -B was submitted to silica gel column chromatography for semiquantitative purpose, and the results obtained are shown in Table II.

Formation of some carbonyl compounds from ASA, D-fructose and D-xylose under the storage condition

ASA (1.76 g), D-fructose (1.8 g) and D-xylose (1.5 g) were dissolved respectively in 200 ml of Clark-Lubs' buffer solution which was deoxygenated before use,