Oxidation of D-(-)-Ribose with H_2O_2 and Lipid Hydroperoxides

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D-(-)-ribose was subjected to oxidation with equimolar amounts of H_2O_2/Fe^{2+} and 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid/Fe^{2+}. Smaller carbohydrates, carbonyl compounds, hydroxy acids and polyols were produced. Identification of carbonyl compounds was achieved by trapping with pentafluorobenzylhydroxylamine hydrochloride (PFBHA-HCl). All products were identified by GC/MS after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA). The reactions were repeated with D-[$^{13}C_1$]-ribose in order to reveal the mechanism of oxidation. A possible reaction pathway is outlined starting by removal of hydrogens from C-H bonds.

Introduction

In a series of model reactions we investigated lipid peroxidation (LPO) of unsaturated fatty acids (Loidl-Stahlhofen and Spiteller, 1994; Loidl-Stahlhofen *et al.*, 1994; Mlakar and Spiteller, 1994; Mlakar and Spiteller, 1996; Herold and Spiteller, 1996) and identified some previously unknown LPO products (Loidl and Spiteller, 1993). Compounds derived by LPO were detected in tissue samples after myocardial infarction (Kumari and Menon, 1987; Meerson *et al.*, 1982; Dudda and Spiteller, 1996) and in LDL of patients suffering from atherosclerosis (Glavind *et al.*, 1952; Dargel, 1992; Esterbauer *et al.*, 1992; Jira and Spiteller, 1996).

The noxious properties of lipid hydroperoxides (LOOH) – no matter whether derived enzymatically or by autoxidation during LPO – are based upon the generation of intermediate radicals like $LO^{\bullet}$ and $^{\bullet}OH$. These are produced from LOOH with Fe^{2+} ions in Fenton-type reactions. The radicals do not only cause further oxidative degradation of LOOH to toxic products, e.g. 4-hydroxynonenal (Benedetti *et al.*, 1980; Pryor and Porter, 1990; Esterbauer *et al.*, 1991), malondialdehyde (MDA) (Esterbauer *et al.*, 1991; Pryor *et al.*, 1976) or α-hydroxyaldehydes (Loidl-Stahlhofen and Spiteller, 1994), but react also with many physiologically important molecules e.g. peptides (Brot and Weissbach, 1993; Nair *et al.*, 1986), purine bases and nucleosides (Lauf, University Bayreuth, personal communication) and also DNA (Brambilla *et al.*, 1989; Inouye, 1984; Vaca *et al.*, 1988). $LO^{\bullet}$ radicals were shown to attack double bonds by epoxidation (Meyer and Spiteller, 1993).

Recently we have shown that LOOH are generated by cell damaging processes (Herold and Spiteller, 1996). Cell damage occurs during many diseases e.g. myocardial infarction or atherosclerosis and also in the course of ageing. Recently 13-HPODE, together with the 9-isomere, was identified in heart tissue after myocardial infarction (Dudda and Spiteller, 1996).

The intention of this investigation was to study the susceptibility of sugar residues to radical reactions initiated by cleavage of LOOH. Decomposition of sugars induced by $OH^{\bullet}$ radicals, produced from H_2O in radiation experiments, was already studied in detail (Schuchmann and von Sonntag, 1977; von Sonntag and Dizdaroglu, 1977; Bothe *et al.*, 1978; von Sonntag, 1980). Since generation of $OH^{\bullet}$ radicals by radiation is not a common pro-

Abbreviations: BHT, 2,6-di-*tert*-butyl-4-methylphenol; EDTA, ethylenediamine-tetraacetic acid disodium salt dihydrate; EI, electron impact; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; LOOH, lipid hydroperoxide(s); LPO, lipid peroxidation; MDA, malondialdehyde; MSTFA, N-methyl-N-trimethylsilyl-trifluoroacetamide; PFBHA-HCl, pentafluorobenzylhydroxylamine hydrochloride; OMHA-HCl, O-methylhydroxylamine hydrochloride; PFB-oxime, pentafluorobenzyl-oxime; TMS, trimethylsilyl; 13-HPODE, 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid.

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cess in Nature, we investigated the degradation of ribose induced by LO• radicals derived by decomposition of LOOH. In contrast to earlier investigations we concentrated our efforts on aldehydic products which were trapped by reaction with pentafluorobenzyl-hydroxylamine or methylhydroxylamine. Since aldehydic products are also produced by decomposition of 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid, we first investigated the aldehydic products obtained by reaction of ribose with •OH radicals, generated from H_2O_2 and Fe^{2+} in a Fenton reaction. Products were analyzed by GC/MS after derivatization. The results enabled us later to recognize similar products in the more complicated reaction mixture obtained after the oxidation of D-(-)-ribose with equimolar amounts of purified 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid.

Materials and Methods

Chemicals

N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey & Nagel (Düren, Germany) and D-[$^{13}C_1$]-ribose from ABCR GmbH (Karlsruhe, Germany). All other chemicals were purchased from Fluka (Neu Ulm, Germany). Solvents, obtained from Merck (Darmstadt, Germany), were distilled before use. TLC was performed with home made 0.75 mm PF_{254} silica gel 60 (Merck Darmstadt, Germany) plates. Linoleic acid was stored at -18°C under argon. 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid was prepared as described previously (Axelrod *et al.*, 1981; Kraus *et al.*, 1991) and purified by HPLC (Teng and Smith, 1985).

Oxidation of D-(-)-ribose and D-[$^{13}C_1$]-ribose with equimolar amounts of Fe^{2+}, H_2O_2 or 13-HPODE

30 μmol D-(-)-ribose (4.5 mg) or D-[$^{13}C_1$]-ribose (4.5 mg) were incubated for 24 h at 37°C with 30 μmol $FeSO_4$-$7H_2O$ (8.3 mg) and either 30 μmol of H_2O_2 (30 μl of a solution of 3% (w/v) H_2O_2 in H_2O) or with 30 μmol 13-HPODE (9.4 mg) in a solution of 23 ml 0.1 M aqueous Na_2HPO_4/KH_2PO_4 -buffer (pH 7.4) and 45 ml 0.15 M aqueous KCl.

The same reactions were carried out without H_2O_2 or 13-HPODE or with these reagents in the absence of Fe^{2+}.

The oxidation experiments were interrupted by addition of 160 μl of a 2% (w/v) methanolic solution of BHT and 5 ml of a 1% (w/v) solution of EDTA in H_2O.

Preparation of O-pentafluorobenzyloxime derivatives of the autoxidation products

Reaction mixtures (68 ml) containing H_2O_2 (30 μmol) were incubated with 6 ml of a 0.05 M methanolic solution of PFBHA-HCl (300 μmol PFBHA-HCl) for 1 h at room temperature. When 13-HPODE was used as oxidant 10 ml of a 0.05 M methanolic solution of PFBHA-HCl (500 μmol) were added to the oxidation solutions.

Preparation of O-methyloxime derivatives

Reaction mixtures (68 ml) containing H_2O_2 (30 μmol) were incubated with 600 μmol O-methylhydroxylamine hydrochloride (OMHA-HCl) (50.1 mg) for 24 h at room temperature. If 13-HPODE was used as oxidant 1 mmol OMHA-HCl (83.5 mg) was added.

The mixtures of the derivatized products were extracted three times with 20 ml $CHCl_3$ or n-butanol or were freeze dried. The solvent was removed in a rotary evaporator at 37°C. The residue was trimethylsilylated with MSTFA followed by product analysis with GC/MS.

Gas chromatography/ mass spectrometry

GC was carried out with a Carlo Erba HRGC 5160 Mega Series chromatograph equipped with a flame ionisation detector, using a DB-1 fused-silica glass capillary column (30 m x 0.32 mm i.d.) and a temperature programme from 80°C to 300°C at 3°C/min. The temperature of the injector and detector was kept at 270°C and 290°C respectively. The carrier gas was hydrogen and the splitting ratio was 1:30. Peak area integration was achieved with a Merck D-2500 integrator.

GC/MS was performed on a Finnigan MAT 95 mass spectrometer connected to a MAT-ISIS data system. EI mass spectra were recorded at an ionization energy of 70 eV. A HP 5980 series II gas chromatograph with a 30 m x 0.3 mm (i.d.) DB-1

fused-silica column was used for sample separation. The carrier gas was hydrogen and the temperature programme was the same as used for GC.

To define the R_i values, *n*-alkanes were coinjected, the number of C-atoms multiplied with 100 and the retention time of detected substances was interpolated linearly.

GC/ MS-characterization

Following MS patterns were measured: (See also Mlakar and Spiteller, 1994; Mlakar *et al.*, 1996; Loidl-Stahlhofen, 1994). *PFB-oxime TMS ether derivative of* D-*(-)-ribose:* GC (DB-1): R_i= 2100; MS [relative intensity (%)] = 73 (75), 103 (75), 147 (35), 181 (15), 189 (20), 205 (20), 217 (75), 277 (10), 307 (100), 326 (15), 528 (5), 618 (5). *Methyloxime TMS ether derivative of* D-*(-)-ribose:* GC (DB-1): R_i=1720; MS [relative intensity (%)] = 73 (70), 103 (70), 147 (40), 205 (20), 217 (80), 307 (100), 364 (5), 467 (5). *PFB-oxime TMS ether derivative of erythrose:* GC (DB-1): R_i=1900; MS [relative intensity (%)] = 73 (80), 103 (15), 147 (50), 181 (15), 205 (100), 350 (5), 352 (5), 426 (5), 516 (5). *Methyloxime TMS ether derivative of erythrose:* GC (DB-1): R_i=1460; MS [relative intensity (%)] = 73 (50), 103 (15), 117 (25), 147 (55), 161 (15), 205 (100), 217 (10), 262 (5), 307 (5), 334 (5), 350 (5). *Methyloxime TMS ether derivative of glyceraldehyde:* GC (DB-1): R_i=1240; MS [relative intensity (%)] = 73 (100), 103 (20), 147 (25), 160 (15) 248 (5), 263 (5). *TMS ether and ester derivative of ribonic acid:* GC (DB-1): R_i=1810; MS [relative intensity (%)] = 73 (100), 103 (35), 147 (55), 189 (20), 205 (20), 217 (45), 292 (60), 307 (15), 333 (20), 421 (5), 423 (5), 511 (5). *TMS ether and ester derivative of erythric acid:* GC (DB-1): R_i=1570; MS [relative intensity (%)] = 73 (100), 103 (15), 117 (20), 147 (65), 205 (25), 217 (15), 220 (20), 245 (5), 292 (35), 319 (5), 352 (5), 409 (5). *TMS ether and ester derivative of glyceric acid:* GC (DB-1): R_i=1340; MS [relative intensity (%)] = 73 (100), 103 (20), 117 (10), 133 (15), 147 (60), 189 (40), 205 (20), 217 (5), 249 (5), 292 (25), 307 (10). *TMS ether and ester derivative of glycolic acid:* GC (DB-1): R_i=1060; MS [relative intensity (%)] = 73 (90), 103 (5), 117 (5), 131 (5), 133 (5), 147 (100), 177 (10), 205 (15). *Methyloxime derivative of dihydroxyacetone:* GC (DB-1): R_i=1220; MS [relative intensity (%)] = 73 (100), 103 (25), 117 (5), 133 (10), 147 (40), 160 (20), 191 (5), 217 (5), 232 (5), 248 (5), 263 (5). *PFB-oxime TMS ether derivative of glyceraldosone:* GC (DB-1): R_i=2130; MS [relative intensity (%)] = 73 (20), 75 (40), 181 (100), 353 (5), 369 (10), 535 (35). *PFB-oxime TMS ether derivative of tetros-3-ulose:* GC (DB-1): R_i=2270; MS [relative intensity (%)] = 73 (100), 103 (45), 117 (20), 181 (85), 326 (5), 441 (20), 471 (5), 549 (5), 637 (5), 652 (5). *PFB-oxime TMS ether derivative of pentos-3-ulose:* GC (DB-1): R_i=2460; MS [relative intensity (%)] = 73 (70), 103 (10), 117 (25), 147 (45), 181 (40), 205 (100), 424 (5), 550 (10), 651 (5), 739 (5), 754 (5). *PFB-oxime TMS ether derivative of pentos-4-ulose:* GC (DB-1): R_i=2490; MS [relative intensity (%)] = 73 (70), 103 (10), 147 (15), 181 (50), 231 (35), 326 (25), 428 (100), 649 (10), 739 (5), 754 (5).

The labelled compounds were found to have the same R_i-values as the unlabelled ones, the peaks of the ^{13}C containing ions in mass spectra were shifted for one mass unit.

Results

Since the reactions were carried out in aqueous solutions, the products had to be extracted with organic solvents. Underivatized carbohydrates are poorly soluble in organic solvents. Therefore the carbonyl products were transformed to O-pentafluorobenzyloximes (PFB-oximes) by addition of PFBHA-HCl. Also O-methyloximes were synthesized, but preliminary experiments revealed that O-methyloxime derivatives of small aldehydic molecules, e.g. glyoxal or malondialdehyde, are partly lost due to high volatility by removal of the solvent. In addition PFBHA-HCl turned out to be reactive enough to transform carbohydrates – which are usually present in the pyranoside or furanoside form in aqueous solutions – completely into the derivatized aldoses (when O-methyloxime derivatization was used we also found pyranosides and furanosides). Characterization of the products by GC/ MS required transformation of the poorly volatile polyhydroxy molecules in a second derivatization step into volatile trimethylsilyl ethers and esters.

D-(-)-ribose is not degraded if treated with an aqueous solution of Fe^{2+} in the presence of oxygen. Almost no reaction occurs in the presence of H_2O_2 or 13-HPODE, if Fe^{2+} is absent. Only a

„Fenton-system", either H_2O_2/ Fe^{2+} or 13-HPODE/ Fe^{2+}, is able to produce radicals ($HO^{\bullet}$ or $LO^{\bullet}$) causing the degradation of D-(-)-ribose or other sugars.

The investigation revealed that nearly the same products are generated by oxidation with LOOH or H_2O_2. But when H_2O_2 was used aldehydes were obviously converted to corresponding acids. Thus oxidation with H_2O_2 produced in addition glycerinic acid, erythronic acid and ribonic acid. These additional reactions are suppressed by use of 13-HPODE instead of H_2O_2. Table I gives a survey of identified products.

The mechanism of ribose degradation was studied by oxidation experiments carried out with D-[$^{13}C_1$]-ribose. It was expected that the mass spectrometric fragmentation products of compounds containing the labelled C-1 atom should be shifted for one mass unit. This shift was observed only in the case of glyoxal and in the case of oxidized carbohydrates containing five C-atoms.

Discussion

Previous investigations to study the oxidative breakdown of ribose used radicals generated mostly by radiolysis of H_2O (Phillips and Criddle, 1962; von Sonntag and Dizdaroglu, 1977). In these processes $^{\bullet}OH$ radicals are produced, which are more reactive than $LO^{\bullet}$ radicals formed by decomposition of fatty acid hydroperoxides in a Fenton type reaction. Since production of $^{\bullet}OH$ radicals by radiolysis of H_2O seems to be a very unusual process in biological tissue we reinvestigated the oxidation of ribose initiated by $^{\bullet}OH$ radicals as well as $LO^{\bullet}$ radicals and compared the product spectrum whether there are differences or not. The investigation focussed on the production of low molecular weight aldehydic compounds which might have escaped from detection until now due to their volatility. A great number of products, already detected after radiolysis of ribose (Phillips and Criddle, 1962; von Sonntag and Dizd-

Table I. Products of radical induced oxidation of D-(-)-ribose.

Identified oxidation products		Reference	Identified oxidation products		Reference
$CH_2(OH)-COOH$	glycolic acid	Phillips 1962	$CH_2(OH)-CH(OH)-CH_2(OH)$	glycerol	
$CH_2(OH)-CH(OH)-COOH$	glyceric acid		$CH_2(OH)-CH(OH)-CH(OH)-CH_2(OH)$	tetritol	
$CH_2(OH)-CH(OH)-CH(OH)-COOH$	erythronic acid	Phillips 1962 von Sonntag 1977	$HOC-CHO$	glyoxal	
			$OHC-CH_2-CHO$	MDA	
$CH_2(OH)-CH(OH)-CH(OH)-CH(OH)-COOH$	ribonic acid	Phillips 1962 von Sonntag 1977	$CH_2(OH)-C(=O)-CH_2(OH)$	dihydroxyacetone	
			$CH_2(OH)-C(=O)-CHO$	glyceraldosone	
$H-CHO$	formaldehyde		$CH_2(OH)-C(=O)-CH_2-CHO$	tetros-3-ulose	
$CH_2(OH)-CHO$	glycolaldehyde	Phillips 1962	$CH_2(OH)-CH(OH)-C(=O)-CH_2-CHO$	pentos-3-ulose	von Sonntag 1977
$CH_2(OH)-CH(OH)-CHO$	glyceraldehyde	von Sonntag 1977	$CH_2(OH)-C(=O)-CH(OH)-CH_2-CHO$	pentos-4-ulose	von Sonntag 1977
$CH_2(OH)-CH(OH)-CH(OH)-CHO$	erythrose	Phillips 1962 von Sonntag 1977			

aroglu, 1977) were recognized in this investigation (Table I). In addition to these products, the applied method – directed particularly to trap aldehydes – revealed the generation of previously unknown aldehydic oxidation products of ribose, e.g. glyoxal. Also glyceraldehyde and glycolaldehyde were detected. Especially the generation of glycolaldehyde is remarkable: α-hydroxyaldehydes lacking additional OH functions at other carbon atoms can not be stabilized like sugars by hemi-acetal formation. They are of high reactivity and were found to react with stimulated macrophages causing an oxidative burst (Heinle, University Tübingen, personal communication). Therefore glycolaldehyde might react in the same manner.

Based on mechanistic studies by von Sonntag (Schuchmann and von Sonntag, 1977; Bothe *et al.*, 1978; von Sonntag, 1980) the genesis of oxidation products from ribose might be visualized as follows:

Hydrogen abstraction from any C-H bond of ribose **1** is induced by $^{\bullet}OH$ or $LO^{\bullet}$ radicals. Removal of the hydrogen in position 2 for instance produces the radical **2** which may add oxygen forming radical **3** (Scheme 1).

We speculate that the peroxy radical may remove a hydrogen atom from any adjacent OH group to generate e.g. **4** or **5** (Scheme 2). These radicals **4** and **5** may suffer decomposition by cleavage of an adjacent C-C bond (Scheme 2). Thus **4** would be able to decompose to the intermediate **6** which is hydrolyzed to glyceraldehyde **7**. Alternatively glyceraldehyde may be produced via the intermediates **5**, **8**, **9** and **10** (Scheme 2).

If radical **8** abstracts hydrogen from another molecule or in an intramolecular reaction, glycerol **11** is obtained after hydrolysis of the hemi-acetal structure. The hydroperoxy acids – produced after hydrolysis – may suffer further cleavage in the presence of Fe^{2+}. Thus aldehydic acids are obtained after hydrogen abstraction.

This hydrogen abstraction occurs obviously at any CH-group. Thus we must conclude that the reaction is not restricted to ribose but also to nucleic acids and might therefore contribute to the damaging effects of LOOH in biological material.

We suppose that in the course of cell injury, in which the Ca^{2+} ion concentration in the cell is increased (Bellomo *et al.*, 1982; Bellomo *et al.*, 1984) enzymes are activated, e.g. lipoxygenases and proteases (Orrenius *et al.*, 1989). Activation of proteases removes the shielding protein cover of iron containing proteins and Fe^{2+} ions are liberated (Aust, 1988). Activation of lipoxygenases induces the formation of hydroperoxides of polyunsaturated fatty acids (Hölzel and Spiteller, 1995) and these produce in a Fenton-like reaction with Fe^{2+} ions free radicals like $LO^{\bullet}$. As a consequence not only unsaturated fatty acids but also sugars may suffer degradation as outlined above, since the local concentration of Fe^{2+} might be sufficient. Saccharides are located on the cell surface and involved in immune defence mechanisms. We speculate that their degradation by radicals might influence the immune response.

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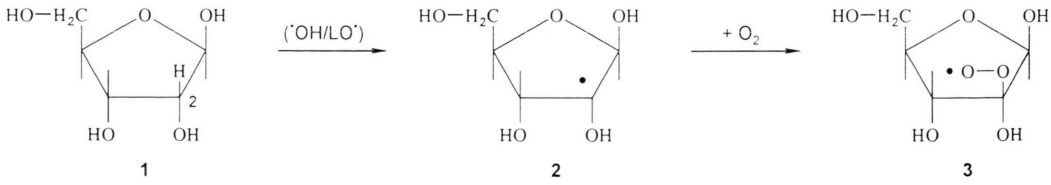
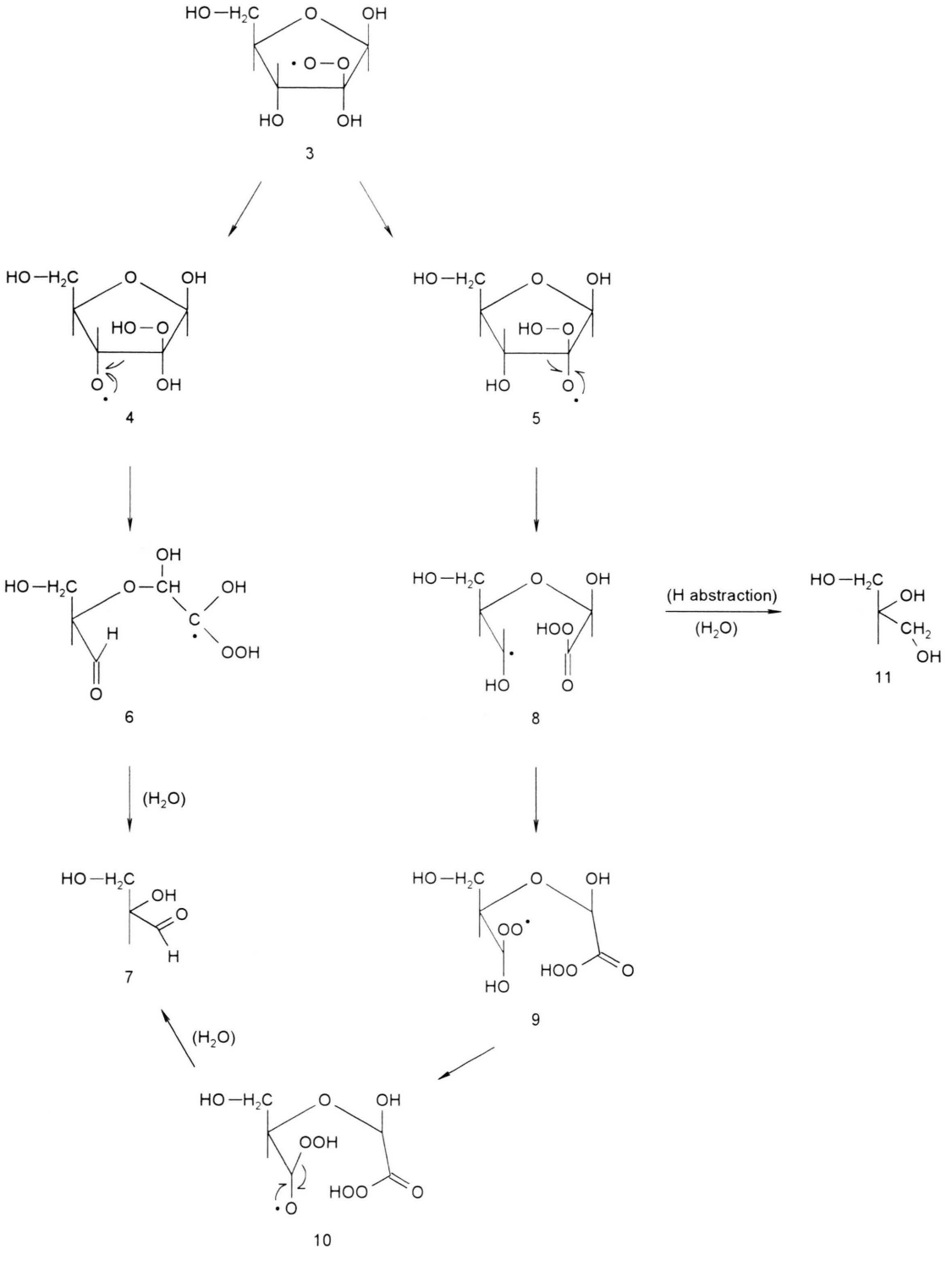


1 2 3

Scheme 1. Possible starting reaction for the radical induced oxidation of ribose.



Scheme 2. Putative pathway for the formation of glyceraldehyde and glycerol.

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