Electron Spin Resonance Spin-Trapping Analysis of γ-Induced Radicals in Polycrystalline α-D-Glucose

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γ-Induced radicals in polycrystalline α-D-glucose are trapped with a water–ethanol solution of 2-nitroso-2-methylpropane. Five long-lived nitroxide spin-adducts are evidenced by electron spin resonance (ESR). Specifically labeled glucoses are also studied. Tentative assignments are discussed for each nitroxide radical.

Introduction

To understand the radiolysis mechanism of starches and thus to ascertain the wholesomeness of such treated polysaccharides and related foods for human consumption, we carried out electron spin resonance (ESR) studies on γ-irradiated starches.[1,2] These ESR powder spectra being unresolved and starch being a too complicated macromolecule,[2] we set out to investigate simple sugars. Glucose oligomers should be the best models[3] but glucose requires its own particular analysis in order to understand better, in the next step, the influence of the glycosidic link on the radiolysis of these different saccharides.

Unfortunately, the ESR signals of glucose irradiated in the powder state[4] are still uninterpretable. Thus, we report here the results obtained with the spin-trapping technique,[5,6] recently carried out elsewhere[7,8] on sugar moieties of nucleic acids. With this powerful method, the "glucose radicals" G·, induced in the powder state, are converted to long-lived nitroxide spin-adducts in the liquid phase by reaction with a spin-trap R—N=O:

$$G\cdot + R{-}N{=}O \rightarrow R{-}\underset{\displaystyle O\cdot}{\overset{\displaystyle |}{N}}{-}G$$

The ESR signals of such nitroxide radicals being persistent and well resolved, their analysis should enable us to identify the structure of the main trapped radiation-induced radicals in glucose.

Experimental Section

Chemicals (Figure 1 and Table I). Simple sugars were purchased from Fluka, Merck, and Prolabo Companies. Specifically labeled glucoses were from Merck Sharp and Dohme for ^2H labeling or were provided by the CEN Saclay (SMM, F91191, Gif-sur-Yvette) for ^{13}C labeling.

The selected spin-trap, 2-nitroso-2-methylpropane (*t*-BuNO or NMP) was from Aldrich Chemical Co. The corresponding deuterated trap (^2H-NMP) was prepared in the laboratory by oxidation of *tert*-butyl-d_9-amine[9] derived from *tert*-butyl-d_9 alcohol (CEN Saclay).

γ Radiolysis. Unless otherwise stated, polycrystalline sugars are irradiated under nitrogen and at room temperature at a dose of 20 kGy. The ^{60}Co γ cell supplies a dose rate of 5.6 kGy h^{-1}.

Spin-Trapping. NMP (60 mg) is first dissolved in 3.3 mL of deoxygenated ethanol. Immediately prior to trapping, 0.3 mL of this solution is added to 0.6 mL of deoxygenated water in the dark and at room temperature. One minute later, the irradiated sugar (usually 30 mg) is trapped by 0.6 mL of the NMP solution.

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TABLE I: Chemical Structure of Studied Sugars with Reference to Glucose[a]

sugar	reversed configuration on carbon	chemical function on C(5)
mannose	C(2)	CH_2OH
galactose	C(4)	CH_2OH
ribose	C(3)	H
arabinose	C(4)	H
glucuronic acid		COOH

[a] See Figure 1 for carbon notation.

TABLE II: ESR Constants of Nitroxide Radicals NMP(A-E) Obtained by Spin-Trapping of Irradiated α-D-Glucose

NMP(...)	g[d]	a_N,[e] G	a_H,[f] G	a_H, G
A	2.0058	16.05	1.6	0.25
B	2.0058	16.05	3.2	0.25
C	2.0058	16.05	4.8	
D	2.0058	15.45	4.6	
E	2.0058	16.05		0.2[c]
(S)[a]	2.0058	16.85		
(H)[b]	2.0060	14.4	13.9	

[a] Radical *t*-Bu-N(O·)-*t*-Bu.[7] [b] Radical *t*-Bu-N(O·)-H.[7] [c] Approximative total coupling. [d] ±0.0001. [e] ±0.05 G. [f] ±0.15 G.

ESR Measurements. ESR signals are recorded on a Bruker 200 D 10 spectrometer connected to an Aspect 2000 computer.

Results

Unlabeled Glucose. As shown in Figure 2, the ESR spectra of the spin-adduct solution are complex and consistent with at least five overlapping signals. Let us designate as NMP(A), NMP(B), ... the nitroxide radicals

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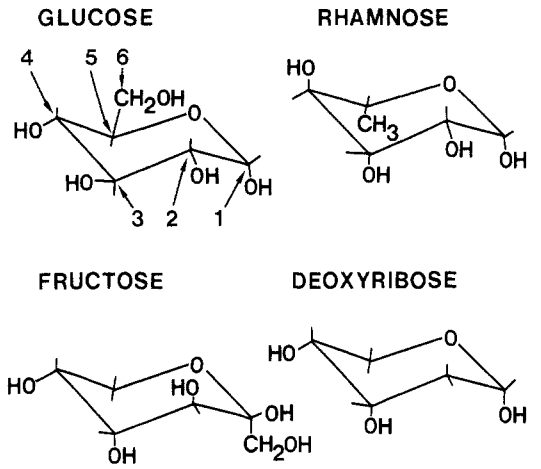


Figure 1. Chemical structure of studied sugars.

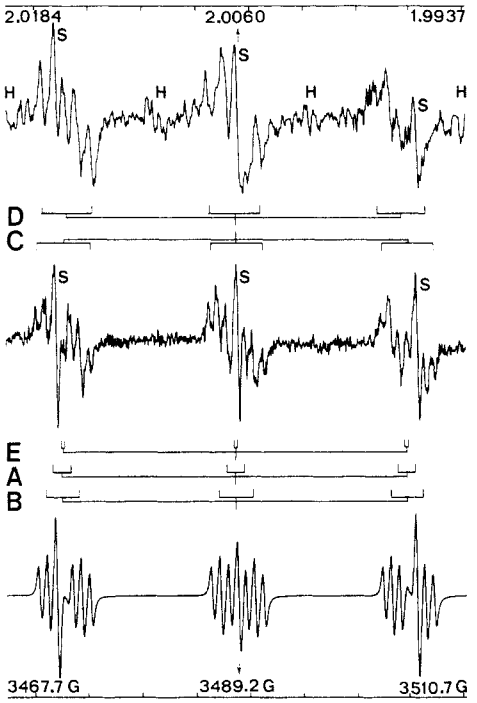


Figure 2. ESR spectra of the spin-adduct of irradiated glucose with ^{2}H-NMP in water–ethanol solution. Lines A, B, ... correspond to nitroxide radicals NMP(A), NMP(B), ...: (top) initial spectrum, recorded just after irradiation and trapping (predominant D-over-C lines); (medium) final spectrum (predominant C-over-D lines) recorded 40 h later; (bottom) simulation of the final spectrum (long-range couplings are neglected) with A, B, C, E (30, 35, 25, 10), and the interference signal S.

generated from sugar radicals induced in the powder state, i.e., G(A), G(B), ...; S and H signals do not derive from sugar radicals but from interfering spin-trap reactions.[7] The g factor and coupling constant values (Table II) were extracted from a series of experimental spectra, by using the Aspect 2000 computer for measurements and simulations. The spectrum analysis requires a high resolution, which is obtained with low-concentration radical solutions. As a consequence, the signal-to-noise ratio is weak, as shown in Figure 2.

Let us emphasize the following:

(1) Spin-trapping with ^{2}H-NMP shows weak, long-range proton couplings for A, B, and E;

(2) Trapping just after irradiation or several days or months after treatment changes the ESR display. The relative concentrations of the observed species vary with time: the decay rate is particularly fast for the D signal,

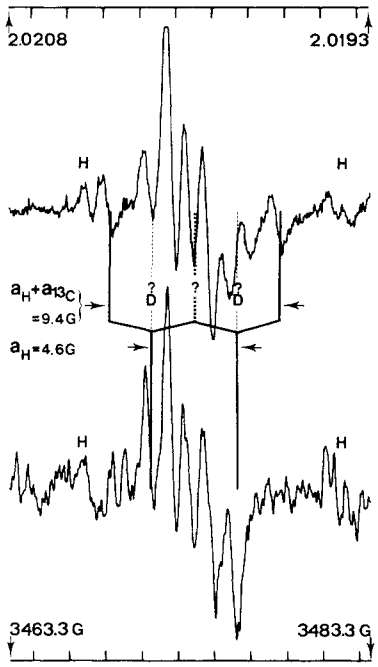


Figure 3. Low-field portion of the ESR spectra of the spin-adducts (^{2}H-NMP in water–ethanol solution) recorded just after irradiation and trapping: (top) ^{13}C(6)-labeled glucose; (bottom) unlabeled glucose. (The corresponding lines, at higher fields, are changed in the same way.)

recorded on the very first spectra only; on the other hand, the A, B, and C lines can still be detected 1 week later. In a typical experiment the changes in the ESR spectra are studied for 1 day. It must be noted that the spin-adduct yield is not readily measurable in our experiments. As shown in a recent report,[5] the spin-trapping efficiency depends on numerous factors: solvent nature, monomer-to-dimer ratio of NMP, particle size, rate of dissolution, ...; consequently, a kinetic study would require a considerable amount of additional experimental work.

(3) Spin-trapping at room temperature of glucose irradiated at low temperature (77 K) allows an unambiguous determination of the D signal, the NMP (D) contribution being important under these conditions. On the other hand, glucose irradiation at room temperature, then heating at 80–90 °C, shows the only presence of NMP(C) between the two radicals—it has been checked that heating nonirradiated glucose induces no signal. Moreover, the C-over-D ratio increases with the times between irradiation, spin-trapping, and recording of the ESR spectrum.

(4) The sugar origin greatly influences the ratio of the different radicals; similar ESR spectra may be obtained with spin-trapping immediately following irradiation or delayed several days. Of course, these "different" glucoses are only unalike in crystallinity. The particle size may strongly influence the spin-adduct efficiency for each radical, as proved recently with a polycrystalline amino acid.[5] In the same way, we have noticed a strong effect of the solvent nature on this efficiency; therefore, we have restricted our investigations to a constant surrounding (water–ethanol, 70/30) without studying the possible solvent effect on hyperfine couplings.

Labeled Glucose. Labeling (^{2}H or ^{13}C) in position 1 does not alter the main couplings of the spectra; however, a 6-labeling significantly modifies the signals. The ABE (S, H) pattern remains unchanged, but the C or D doublets (of triplets) are lacking:

(1) C and D are likely converted to unresolved singlets (of triplets) in the case of ^{2}H labeling.

(2) In the case of ^{13}C labeling the spectrum, recorded just after irradiation and trapping, shows, besides the ABE (S,

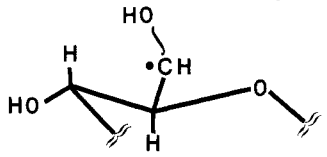
H) components, several new lines (Figure 3). Two of them have the same a_N and g factor as the D signal; they presumably result from the combined interaction of the ^{13}C with the corresponding H atoms. In this hypothesis, the measured splitting (9.4 G) is equal to $a_{^{13}C} + a_H$ and gives, a_H(D) being equal to 4.6 G, a coupling constant $a_{^{13}C}$ (D) = 4.8 G. However, this assumption implies in each set of lines an additional degenerate central line, unfortunately superimposed on the E, S + E, or A line. Moreover, the α and β anomers were both present in approximately equal proportions in our labeled glucoses; consequently, the spectra interpretation is inconvenienced in the case of glucose studied just after irradiation ("D form") and even completely prevented in the case of glucose trapped later ("C form").

Other Simple Sugars. Kuwabara et al.[7] already trapped several simple sugars (glucose, mannose, fructose, deoxyribose, and ribose). They eventually saw lines A–C but did not notice the presence of D and E. We studied these sugars again and then arabinose, rhamnose, and glucuronic acid (Table I and Figure 1); we only observed C and D when the C(6)H_2OH group exists. The particular case of galactose, which possesses this group but displays neither C nor D signal, will be discussed in the following.

Discussion

Assignment of Species G(C) and G(D). The large doublet (of triplet) (a_H = 4.8–5.6 G) observed by Kuwabara et al.[7] apparently corresponds to our signal C; they assigned it to the radical induced by an H abstraction on carbon 6 (structure I), mentioning the fact that C is not detected

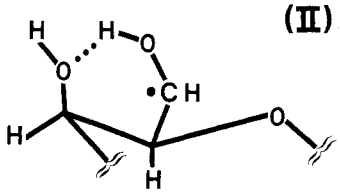
(I)



when the sugar has no C(6)H_2OH group. We had to check this latter hypothesis since the radical NMP(D), not seen by these authors, and NMP(C) have close a_H values.

A first point was that C and D lines were not detected in the case of galactose. However, Gilbert's group[10] described the radicals obtained from the reactions of glucose or galactose with hydroxyl radicals generated in a flow system. They observed an intense ESR signal from the C(6) radical of galactose, compared to glucose, with an enhanced value of $a(\beta$-H) due to the axial 4-OH group. An internal hydrogen bonding, involving the 4- and 6-hydroxyl groups, is suggested to stabilize the conformation of structure II; this would inhibit the spin-trapping and

(II)



consequently explain the absence of C and/or D lines in galactose ESR spectra. Such an internal hydrogen bonding seems also to exist in galactose solutions, if we consider the exceptionally low ^{17}O chemical shift of the 4-OH group of D-galactose with regard to the ones of glucose and relative compounds.[11]

A second point was the precise assignment of G(C) and G(D). Our glucose-labeling data are consistent with the hypothesis of two distinct hydroxyalkyl radicals centered on carbon C(6): the C and D signals are strongly affected by a ^{13}C or ^2H labeling on position 6. Such two distinct radicals were not unexpected as Madden et al.[4] have recently identified, in X-irradiated glucose single crystals, two similar species, but one of which is unstable above 200 K. However, the completely different surrounding conditions preclude any direct correlation between the two Madden C(6) radicals and our C and D signals. The deep influence of the experimental conditions on the D-over-C ratio is in favor of two different stabilities for "our" two C(6) radicals, whose kinetics vary largely with a lot of factors: crystallinity effect (D-over-C ratio depending on the particle size), irradiation at lower temperature (leading to a strong D signal), effect of postirradiation or post-spin-trapping times (allowing or not the D-signal recording). But if two individual conformers are conceivable in the solid state, they never have been observed in solutions of α-glucose. Gilbert et al. only suggested such an occurrence for restricted rotation in this type of radical for β-glucose.[10] Nevertheless, it is hardly credible that two such conformers do not convert into the same nitroxide radical under our conditions. On the other hand, two observed nitroxide radical conformers may correspond to only one sugar radical. This unexpected experimental point might be elucidated later by theoretical calculations.

Assignment of Radical G(E). The very low value of the hyperfine coupling constant (Table II) makes us think that there is no H atom nearby the electron orbital and that G(E) results probably from an H abstraction on carbon 1, 2, 3, 4, or 5. The signal of $^{13}C(1)$-labeled and -unlabeled glucose being roughly similar, we can eliminate position 1. Madden[4] showed that the C(5)-centered hydroxyalkyl radical, easily observed in methylglucose, is not stable in glucose. So, only the positions 2, 3, and 4 are still possible. The H abstraction on C(2) was proved to occur during OH· attack[10] and proposed, if not directly observed, in X-irradiated single crystals of glucose.[4] Moreover, the mannose and glucose spectra are identical, in spite of the OH inversion in position 2; indeed, the H abstraction on C(2) removes the only difference between the two sugars.

However, if this assignment is right, the expected influence of a C(1) labeling on the E signal was not seen in the first recordings. A careful investigation of the only E line area was undertaken by using ^2H-NMP and combining numerous acurate sweeps (1024 points per 10 G instead of 2048 per 70 G). It shows a weak but well-reproducible multiple structure corresponding to long-range couplings (0.1–0.2 G) in the case of unlabeled glucose (Figure 4a), a structure which seems to vanish in the two C(1)-labeled sugars (Figure 4b,c). Tentative spectral simulations indicate the disappearance of the hyperfine structure with introduction of an additional ^{13}C coupling and, to a lesser extent, when replacing one proton by one deuterium (Figure 4d–f).

These factors all suggest that radical G(E) derives from glucose by an H abstraction on C(2).

Tentative Assignments of G(A) and G(B) Radicals. The medium values of their coupling constants a_H (Table II) may correspond to β- or γ-H atoms. Thus, many radical structures are possible until new information can be obtained, for instance, from other specific labeled compounds. the two assignments proposed below support the presence of A or B lines for the different sugars studied here and

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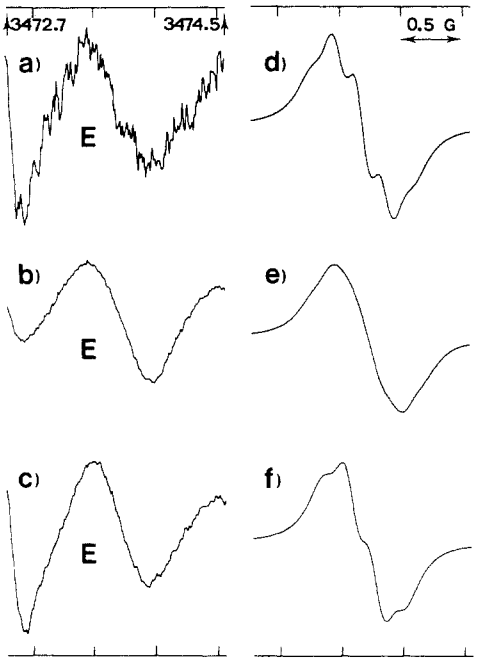
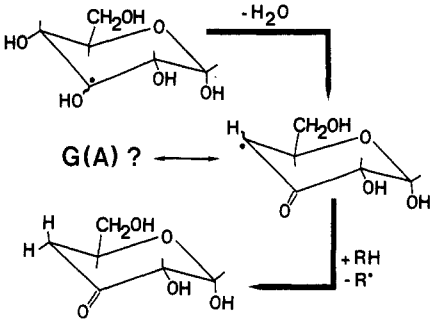


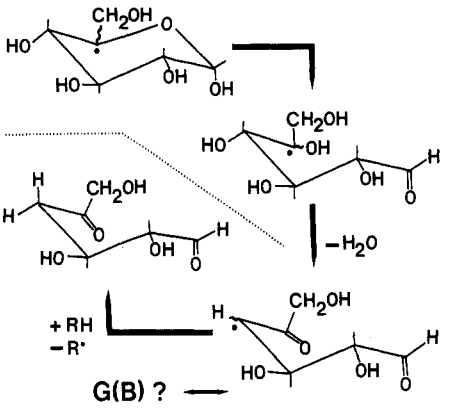
Figure 4. Structure of the E line (spin-trapping of glucose with ^{2}H-NMP). The three left spectra are the combination of 11–14 experimental recording with coefficients chosen so as to get an equal contribution of each spectrum to E line: (a) unlabeled glucose, (b) ^{13}C(1)-labeled glucose, (c) ^{2}H-C(1)-labeled glucose. The right spectra are simulations: (d) four equivalent protons with a_H = 0.18 G, line width = 0.18 G; (e) same as d with an additional ^{13}C coupling of 0.06 G; (f) same as d but with one deuterium (a = 0.028 G) in place of one of the four protons.

Scheme I



elsewhere;[7,10] moreover, they can be intermediates in the formation of two well-known radiolysis products as reported by Von Sonntag:[12] (1) 4-Deoxy-D-*erythro*-3-hexulose was suggested to be induced according to Scheme I, where we associate the intermediate radical with G(A). (2) The formation of 4-deoxy-L-*threo*-5-hexulose is depicted in Scheme II. The last intermediate radical may be related to G(B).

Scheme II



These two schemes have been put forth by Von Sonntag[12] and also described by Norman and Pritchett[13] as an acid- and base-catalyzed reaction in solution: moreover, Gilbert's group[10] obtained evidence for the initial radicals by OH· attack of glucose solutions and even, in low pH medium, the so-called G(A) radical.

Conclusion

The analysis of the structure of the ESR spectrum, and of its time dependence, allowed the assignments of the five spin-adducts observed here. New investigations on glucose, in the α or β form, in a dry or moist state, or with different labeling, are in progress. Lastly, experiments on glucose oligomers, selected as models for starch, should provide significant information on the nature of the free radicals induced by radiation in oligosaccharides and, thus, on the starch radiolysis mechanism.

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