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Free Radicals in γ-Irradiated Single Crystals of Trehalose Dihydrate and Sucrose Studied by Electron Paramagnetic Resonance

ASTRID GRÄSLUND[a] and GÖRAN LÖFROTH[b]

[a] Biophysics Department, Arrhenius Laboratory, University of Stockholm, S-104 05 Stockholm, Sweden, and [b] Radiobiology Department, University of Stockholm, Wallenberg Laboratory, S-104 05 Stockholm, Sweden

The γ-induced free radicals in single crystals of trehalose dihydrate and sucrose have been studied by Electron Paramagnetic Resonance (EPR). In trehalose dihydrate a free radical conversion was observed. Interpretation of the EPR spectra leads to the proposal that the primary radicals are formed by hydrogen abstraction at particular positions in the monosaccharide units, and the secondary radicals are formed by water elimination from the primary radicals. The EPR spectra from the sucrose single crystals may be assigned to free radicals similar to the secondary radicals in trehalose dihydrate.

The free radicals induced by ionizing radiation in disaccharides have been the subject of several Electron Paramagnetic Resonance (EPR) studies in the past.[1–6] In the present investigation we have studied the radiation-induced free radicals in single crystals of the disaccharides trehalose dihydrate and sucrose. In the trehalose dihydrate crystals a 1:1 free radical conversion takes place upon storage at room temperature. Interpretation of the EPR spectra before and after this conversion gave us enough information to propose mechanisms both for the initial radical formation and the subsequent conversion reaction. To our knowledge these free radicals have not been previously identified.

Irradiated sucrose was one of the first substances studied in single crystal form by the EPR method.[1–3] In spite of this the nature of the free radicals has never been adequately explained. Similarities in the EPR spectra from the secondary free radicals in trehalose dihydrate and the free radicals observed in sucrose has led us to conclude that the mechanism of radical formation may be similar in both crystals but that the radical conversion takes place instantaneously in the irradiated sucrose crystal.

EXPERIMENTAL

Single crystals of trehalose dihydrate and sucrose were obtained by dissolving the carbohydrates in H_2O or D_2O followed by addition of ethanol and subsequent storage of the solution at 4 °C until suitable single crystals could be harvested. The solutions had the following compositions: 2.5 g trehalose dihydrate in 31 ml H_2O and 72 ml ethanol or 28 ml D_2O and 53 ml ethanol (corresponding to a ratio of dissociable [D] and [H] of [D]/[H]$\simeq$3:1); 20 g sucrose in 17 ml H_2O and 25 ml ethanol.

The disaccharide materials and their radiation degradation have previously been described.[7] The crystals were irradiated in a ^{60}Co γ-source at 77 K or at room temperature with doses in the range $5 \times 10^{19} - 15 \times 10^{19}$ eV/g.

Figs. 1 and 2 show molecular structures and schematic crystal shapes for trehalose dihydrate and sucrose, respectively. Crystal structures are known both for trehalose dihydrate,[8–10] and sucrose.[11–13] For trehalose dihydrate the space group is $P2_12_12_1$, and 4 formula units are contained in each orthorhombic unit cell with dimensions $a = 17.90$ Å, $b = 12.21$ Å, and $c = 7.59$ Å. For sucrose the space group is $P2_1$, and 2 formula units are contained in each monoclinic unit cell with dimensions $a = 10.86$ Å, $b = 8.70$ Å, and $c = 7.76$ Å. The unit cell axes directions were determined by X-ray crystallography for both disaccharide crystals. Orthogonal sets of axes, *abc* for trehalose dihydrate, and *a*bc* for sucrose, are indicated in Figs. 1 and 2.

For the room temperature studies the crystals

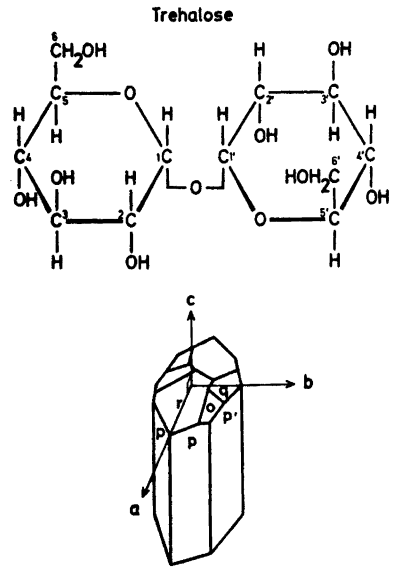


Fig. 1. Molecular structure and schematic crystal shape of the trehalose dihydrate single crystal. The orthogonal *a*, *b*, and *c* directions of the orthorhombic unit cell are indicated.

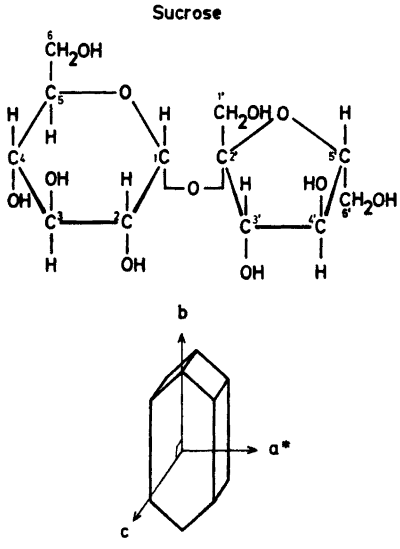


Fig. 2. Molecular structure and schematic crystal shape of the sucrose single crystal. The orthogonal *a**, *b*, and *c* directions of the monoclinic unit cell are indicated.

were mounted in a simple goniometer permitting rotation in a plane perpendicular to the plane of rotation of the spectrometer magnet. For a low temperature recording the sample was simply placed in an EPR tube with a specific direction perpendicular to the magnet plane. The EPR spectra were recorded on a Varian V-4502 EPR spectrometer equipped with 100 kHz field modulation, a 30 cm magnet and a V-4533 cylindrical cavity, around which the spectrometer magnet could be rotated. A modified microwave bridge permitted studies at low microwave power.

EPR spectra at room temperature were recorded in various directions in the main crystallographic planes of the single crystals. Any chosen direction of the magnetic field *H versus* the crystal could be obtained by rotating the goniometer and the spectrometer magnet. In all experiments the microwave power level was chosen as high as possible without causing noticeable microwave power saturation.

All spectra were recorded as first derivatives of the absorption lines. For the quantitative analyses in trehalose dihydrate double integrations were performed on the spectra from polycrystalline samples. This was done by an IBM 360-75 computer, which was fed digitized spectra on paper-tape from a Time-Averaging-Computer (TMC-CAT-1024) connected to the spectrometer and with an attached paper-tape punch.

Dihedral angles in the molecules were calculated by using an IBM 1800 and a Varian 620-i computer.

RESULTS AND DISCUSSION

Trehalose dihydrate, radical A. Fig. 3 shows an EPR spectrum of a single crystal of trehalose $2D_2O$ at 77 K after γ-irradiation at 77 K. The

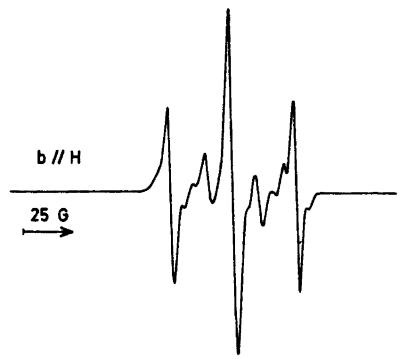
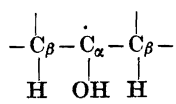


Fig. 3. The EPR spectrum recorded with H||b of a single crystal of trehalose.$2D_2O$ at 77 K after γ-irradiation at 77 K. The field modulation was 0.9 G and the microwave power was 3 μW.

degree of deuteriation was estimated to 75 atom % from the relative amounts of dissociable [D] and [H] in the solutions from which the crystals were precipitated. The partial substitution of dissociable hydrogens with deuterium does not change the EPR spectrum, except for a sharpening of the lines and the diminishing of the weak satellite lines ("shoulders"), which are seen in irradiated non-deuteriated carbohydrates and are probably due to forbidden transitions.[14] The main feature of the spectrum is a 1:2:1 triplet structure with a spacing of 32 G, which is almost isotropic when the sample orientation in the magnetic field is varied. Single crystals irradiated and studied at room temperature yield similar spectra immediately after the irradiation.

The isotropic triplet structure in the EPR spectrum indicates interaction between the unpaired electron and two equivalent protons in β positions (α denotes the carbon atom carrying the unpaired electron, and β its nearest neighbours). The radical A is consistent with the EPR spectrum.



Radical A

From the magnitude of the isotropic coupling it is possible to estimate the dihedral angle θ between the z-direction of the $2p_z$-orbital of the unpaired electron and the $C_\beta - H_\beta$ bond, projected perpendicularly to the $C_\alpha - C_\beta$-bond. Formula (1) has been proposed.[15]

$$A(H_\beta) = B_0 + B_2 \cos^2\theta \qquad (1)$$

where B_0 is small (0 – 3 G) and B_2 is approximately 50 G.[16] $A(H_\beta) = 32$ G corresponds to $|\cos\theta_A| = 0.80 - 0.76$ and $\theta_A \simeq 37 - 40°$ (using $B_0 =$ 0 or 3 G, and $B_2 = 50$ G).

Trehalose dihydrate, radical B. When the irradiated trehalose dihydrate crystal was kept at room temperature for 3 months, a free radical conversion took place in the sample. This was indicated by a complete change of the EPR spectrum. However, double integration of spectra from polycrystalline samples at various times after the irradiation showed that the spin concentration was constant during the reaction. The rate of radical conversion could be increased by warming the sample. The secondary radicals were stable for at least a year at room temperature (as long as the crystal was kept).

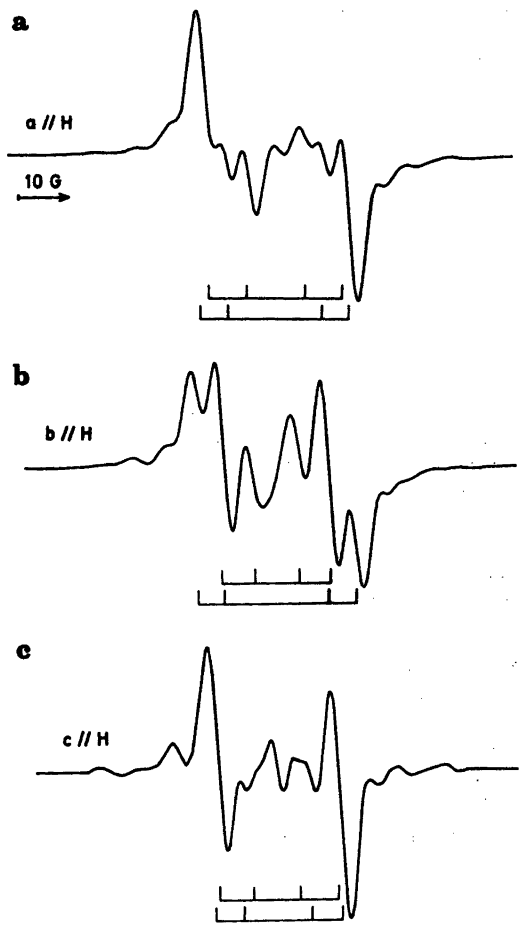


Fig. 4. EPR spectra of a single crystal of trehalose.$2D_2O$ at room temperature after γ-irradiation at 77 K and storage at +40 °C for 3 days. The field modulation was 0.6 G and the microwave power was 30 μW. (a) $H||a$; (b) $H||b$; (c) $H||c$.

The EPR spectrum after the storage was narrower, and of a more anisotropic nature. Fig. 4 shows spectra after the conversion, which were obtained with the magnetic field of the spectrometer in the a, b and c directions (defined in Fig. 1). In these directions all four molecules in the unit cell are magnetically equivalent. In other directions the spectra are generally less

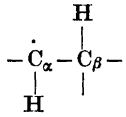
well-resolved, and show considerable asymmetry.

The spectra of Fig. 4 are almost, but not quite, symmetric. We have found it difficult to assign them to one single radical species, responsible for the line pattern in all three directions. Since trehalose dihydrate is composed of two almost identical monosaccharide units, we instead propose that the spectra should be explained as composed of two similar spectral components present in equal amounts, one from each glucopyranose unit of the disaccharide. These two units are magnetically inequivalent also in the directions of the crystallographic axes. Each unit should contribute a quartet of lines, arising from a large anisotropic hyperfine coupling from a hydrogen nucleus in α position and a smaller isotropic one from a hydrogen nucleus in β position. Stick spectra in agreement with this interpretation are inserted below the spectra of Fig. 4.

The isotropic hyperfine coupling $A(H_\beta)$ is approximately 6 G, whereas the anisotropic $A(H\alpha)$ varies, and takes values as low as 14 G and as high as 25 G. (14 G and 25 G can be estimated from Fig. 4b, where the two components are easy to distinguish.)

The magnitude of $A(H_\alpha)$ and its variation is in good agreement with what is usually observed for hydrogen nuclei in α positions. From the magnitude of $A(H_\beta)$ one can estimate the dihedral angle θ in the same way as for radical A [see eqn. (1)]: $A(H_\beta) \simeq 6$ G corresponds to $|\cos\theta_B| = 0.35$ and $\theta_B = 70°$ (using $B_0 = 0$ and $B_2 = 50$ G). Because $A(H_\beta)$ is small, the influence of a small positive value of B_0 in eqn. (1) is comparatively large, and another estimate is $|\cos\theta_B| = 0.25$ and $\theta_B = 75°$ (using $B_0 = 3$ G and $B_2 = 50$ G).

This interpretation of the EPR spectra of radical B leads to the following radical model:



Radical B

The g-values of both radicals A and B are close to 2, and show only a small anisotropy, as expected for carbon-centered free radicals.

Radical formation scheme in trehalose dihydrate. The initial formation of radical A can be simply explained by hydrogen abstraction from one of the carbon atoms in the ring. Radical B shows a H_α hyperfine coupling. Thus the radical conversion from A to B must involve more than a conformational change. The elimination of water from radical A would yield secondary radicals with one H_α and one H_β hyperfine coupling, as observed for radical B. Furthermore, the elimination of water is a plausible radical conversion mechanism. This mechanism has been found to explain the radiomimetic oxidation of monosaccharides in aqueous solu-

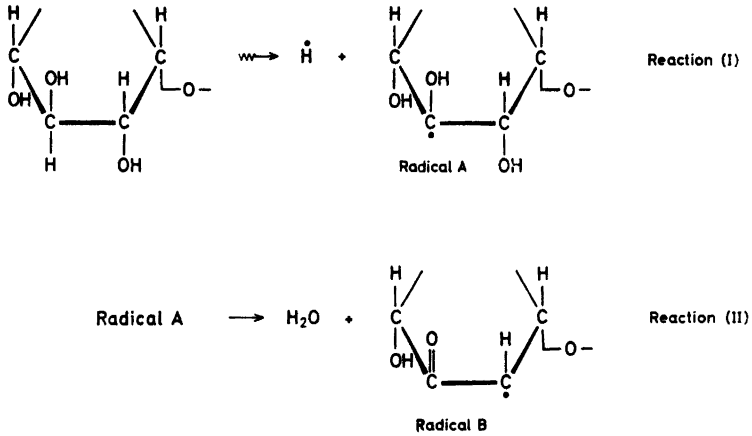


Fig. 5. Schematic model of free radical formation by hydrogen abstraction (I) and conversion by water elimination (II) in the trehalose dihydrate crystal.

Table 1. Calculated values of $|\cos\theta|$, where θ is the dihedral angle, in the trehalose dihydrate and sucrose crystals under assumption of various sites of radical formation. The crystal structures were defined by the atomic coordinates given in Ref. 10 for trehalose dihydrate and in Ref. 13 for sucrose. The calculations were performed in two different ways (a) and (b), assuming different molecular conformations illustrated in Fig. 6 and described in the text. The configurations best corresponding to the experimental $|\cos\theta|$-values ($|\cos\theta_A| = 0.76 - 0.80$ and $|\cos\theta_B| = 0.25 - 0.35$) are set in italics.

Crystal	Site of radical A	H_β	$\|\cos\theta\|$ Calc. (a)	Calc. (b)	Site of radical B	H_β	$\|\cos\theta\|$ Calc. (a)	Calc. (b)
Trehalose dihydrate	C(2)	H(1)	0.03	0.38				
		H(3)	0.85	0.69	C(3)	H(4)	0.89	0.48
	C(3)	*H(2)*	*0.81*	*0.76*	*C(2)*	*H(1)*	*0.03*	*0.24*
		H(4)	*0.89*	*0.71*	C(4)	H(5)	0.89	0.48
	C(4)	H(3)	0.87	0.70	C(3)	H(2)	0.81	0.48
		H(5)	0.89	0.71				
	C(2′)	H(1′)	0.10	0.47				
		H(3′)	0.85	0.72	C(3′)	H(4′)	0.89	0.47
	C(3′)	*H(2′)*	*0.81*	*0.71*	*C(2′)*	*H(1′)*	*0.10*	*0.28*
		H(4′)	*0.89*	*0.70*	C(4′)	H(5′)	0.86	0.52
	C(4′)	H(3′)	0.87	0.71	C(3′)	H(2′)	0.81	0.48
		H(5′)	0.86	0.73				
Sucrose	C(2)	H(1)	0.15	0.51				
		H(3)	0.83	0.70	C(3)	H(4)	0.85	0.48
	C(3)	H(2)	0.83	0.74	*C(2)*	*H(1)*	*0.15*	*0.32*
		H(4)	0.85	0.73	C(4)	H(5)	0.85	0.51
	C(4)	H(3)	0.84	0.71	C(3)	H(2)	0.83	0.49
		H(5)	0.85	0.71				
	C(3′)	H(4′)	0.97	0.71	*C(4′)*	*H(5′)*	*0.70*	*0.50*

tion,[17] and also the radiation-induced chain reactions in crystalline α-lactose monohydrate.[18]

From these considerations we propose a model, summarized in Fig. 5, for the radiation-induced free radical formation and conversion in solid trehalose dihydrate. In Fig. 5 C(2) and C(3) have been chosen as sites of radicals A and B, respectively. The reasons for this assignment will be given later. Reactions (I) and (II) should occur with equal probability in each of the two monosaccharide units.

Because of the complexity of the anisotropic spectrum of radical B we found it difficult to estimate the directions of the principal axes of the $A(H_\alpha)$ tensors and in this way determine which carbon atoms carry the unpaired electrons in radicals A and B. However, the values of $A(H_\beta)$ are related to the molecular configuration *via* eqn. (1). The experimentally estimated values of the dihedral angle θ can be compared with calculated values of θ based on the fractional atomic coordinates given in Ref. 10. Since the conformational changes accompanying free radical formation are unknown, and probably depend also on the hydrogen bonding in the structure, this can only be done approximately and be used to confirm numerically the results from visual inspection of molecular models. Only carbon atoms C(2), C(3), and C(4) and the corresponding C(2′), C(3′), and C(4′) are possible sites of radical A, if the proposed mechanism of radical formation and conversion is valid. Initial radical formation at C(1) and C(1′) would not give the proper configuration

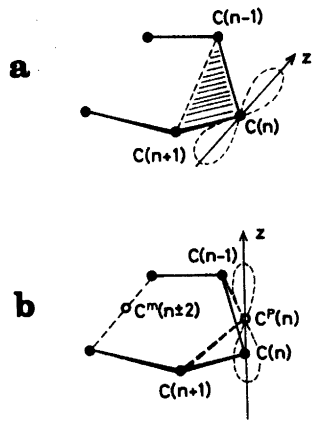


Fig. 6. Schematic configurations of a free radical at C(n). The direction of the z orbital axis of the unpaired electron in radical A is indicated.
(a) The monosaccharide ring system is assumed unchanged.
(b) C(n) is assumed to move into a new position $C^P(n)$ in a plane defined by C($n+1$), C($n-1$) and the mean value $C^m(n\pm2)$ of C($n+2$) and C($n-2$). Radical B assumed to be positioned at C($n+1$) or C($n-1$), has its radical plane defined by C($n-2$), C($n+2$), and C($n-1$) or C($n+1$), respectively.

for radical A. C(5) and C(5′) do not have the OH group necessary for the water elimination mechanism.

As a start the fractional atomic coordinates were transformed to space coordinates in Ångström units. The directions of the orthorhombic unit cell axes were used as an orthogonal coordinate system. The calculations were then performed in two ways, tentatively chosen after building molecular models (Fig. 6): (a) We assumed that the configuration of the monosaccharide ring was intact, and that the z orbital axis of the unpaired electron at C(n) ($n = 2, 3, 4, 2', 3'$ or $4'$) was perpendicular to the plane defined by C($n+1$), C(n) and C($n-1$). (b) We assumed that the initial site of radical formation C(n) was twisted into a new position $C^P(n)$ in a radical plane defined by C($n+1$), C($n-1$) and the mean value of C($n+2$) and C($n-2$), denoted $C^m(n\pm2)$. (The notation C($n+2$) or C($n-2$) may also denote the ring oxygen atom). After the conversion reaction, when C($n+1$) or C($n-1$) is the site of the unpaired electron, the radical plane was assumed to

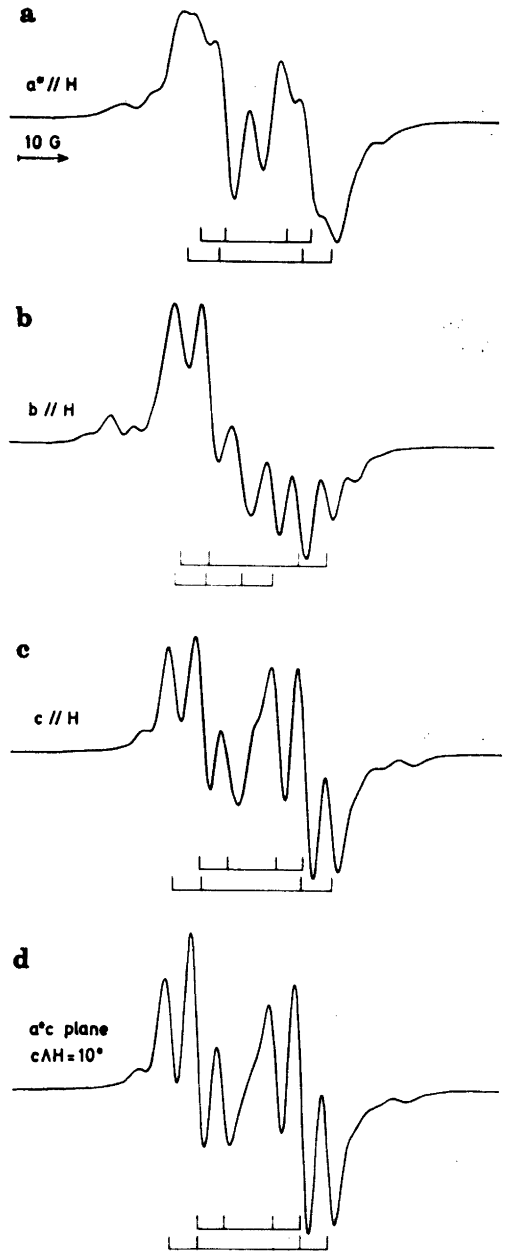


Fig. 7. EPR spectra of a single crystal of sucrose at room temperature after γ-irradiation at room temperature. The field modulation was 1.7 G and the microwave power was 0.3 mW.
(a) $H \| a^*$; (b) $H \| b$; (c) $H \| c$; (d) H in the a^*c plane, and the angle between the c axis and H is 10°.

remain approximately the same, and was defined by C($n+2$), C($n-2$) and C($n-1$) or C($n+1$), or the corresponding atoms in the second monosaccharide ring.

The results of these calculations, both yielding similar results, are summarized in Table 1. A comparison with the experimentally estimated values of $\cos\theta_A = 0.76-0.80$ and $\cos\theta_B = 0.25-0.35$ indicates that the most probable sites of radical A are C(3) and C(3′), which after the elimination of water yield radicals B positioned at C(2) and C(2′). This is also in agreement with what one can guess after inspection of molecular models. It can be observed that calculation (b) seems to account better for the experimental situation than does calculation (a).

The stable radical in sucrose. Fig. 7a–c shows the EPR spectra in the a^*, b, and c directions of a sucrose single crystal, γ-irradiated at room temperature. Similar spectra are observed from crystals irradiated and investigated at 77 K,[3] and from crystals grown in deuteriated media.[2] The spectra remain essentially stable for years. Because of the crystal symmetry, the two molecules in each unit cell should be magnetically equivalent in the a^*-c plane and in the b direction. However, the presented EPR spectra (which are similar to, but better resolved than the spectra shown in Ref. 1 for the corresponding directions) are clearly asymmetric. This must be due to g-value anisotropy and at least two contributing radicals with different orientations. The spectrum of Fig. 7c in the c direction is almost identical to the trehalose radical B spectrum of Fig. 4b. The best resolved spectrum in the a^*c plane, shown in Fig. 7d, was obtained in a direction at 10° angle from the c axis. This spectrum is also very nearly symmetric.

The symmetric spectrum agrees with the earlier postulated radical [2,3] in sucrose which involves hyperfine couplings to one H_α and two identical H_β's with small couplings. The EPR spectrum should then be a double 1:2:1 triplet. However, that model does not explain the observed anisotropies in the spectra.

Instead, in view of the similarities between the stable sucrose EPR spectrum and that of the stable radical B of trehalose dihydrate, we propose that the stable sucrose radicals are similar to the radicals B of trehalose dihydrate. Thus there should be one radical component with one H_α and one H_β hyperfine coupling in each of the two monosaccharide units, which are magnetically inequivalent also in the a^*c-plane and in the b direction. Stick spectra according to this interpretation are inserted in Fig. 7. It is reasonable to assume that the reaction routes have been similar in the two disaccharides, but that the radical A stage is much less stable in sucrose, so that only the water elimination radical is observed.

The value of A(H_β) is practically identical to that found for radical B in trehalose dihydrate. Thus we obtain $|\cos\theta_B| \simeq 0.25-0.35$. Calculations of dihedral angles θ based on the atomic coordinates given in Ref. 13 were performed in the same way as was done for trehalose dihydrate. Since the sucrose unit cell is monoclinic, the orthogonal coordinate system for the space coordinates was chosen as a^*bc.

The results of the calculations are shown in Table 1. In the glucopyranose ring the calculated values are similar to those from trehalose dihydrate, and C(2) is indicated as the site of the unpaired electron. In the fructofuranose ring there is only one possibility for the proposed mechanism to work, *i.e.* carbonyl formation at C(3′) and the stable radical centered at C(4′). However, the calculated value of $\cos\theta$ seems too large to agree with the experimentally estimated value. The reason could be a more fundamental conformational change than the one we have considered.

In studies of the radical processes in frozen aqueous solutions of sucrose and D-glucose, Baugh *et al.*[19] observed two types of radicals originating from the solutes. One was described as a 1:2:1 triplet with spacing of 29.5 G, the other one as a 1:1 doublet with a spacing of 20.5 G. These values of coupling constants suggest that these powder spectra correspond to radicals similar to those described as radicals A and B in the present study. In a powder spectrum one does not expect the small H_β coupling of radical B to be resolved.

The assignment of radicals to both monosaccharide units in sucrose is in agreement with a recent ENDOR study,[20] in which evidence is put forward that protons from both units are involved in forming the so called matrix ENDOR signal in the irradiated samples.

Comparison with radiochemical data. The present interpretation of the radical structures in trehalose dihydrate and sucrose is in line

with previously reported chemical data. The result that both monosaccharide moieties harbour radicals complies with the fact that both monosaccharide moieties in sucrose are radiation-damaged to a similar extent.[7] The result that the radicals are associated with C(3), C(3′), C(2), and C(2)′ is in accord with the chemical analysis showing that not more than a small part of the radiation induced degradation is linked with breakage of the glycosidic bond in the investigated disaccharides.[21] It is also in accord with the result that the yield of acids is small compared with the total degradation.[7,22] Formation of neutral carbonyl compounds which is indicated in the present study has also been observed in chemical analyses.[23,24]

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