ESR Investigations on Lyophilized Blood: Mixtures with Ascorbic Acid

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The main contribution to ESR spectra of lyophilized tissues of plant or animal origin usually is given by an anisotropic singlet, which is known to be caused at least in the majority of cases by the adsorption of an ascorbyl radical. Model systems have been investigated in order to discuss experimental parameters, which influence these spectra qualitatively and quantitatively.

Introduction

Electron spin resonance (ESR) investigations of lyophilized samples have been used in a large number of studies on the role of free radicals in cancer [1, 2]. Strong correlations between concentrations of free radicals and cancer have been reported. These results include increases in free radicals very early in tumor development and radical changes in lyophilized blood in cancer patients [3–5]. Although studies of this type are of potential usefulness, there is considerable controversy over the validity and significance of those findings [2, 7].

Several fundamental unanswered questions on the interpretation of the data subsist, which concern the relationship between the ESR signals in lyophilized tissues and the radical yield before lyophilization and the reasons for the high degree of variability in the ESR signals after lyophilization. The main contribution to the signal is usually represented by an asymmetric singlet attributed to the ascorbyl radical [2, 6, 8].

In this paper results on model systems are presented documenting the strong dependence of the fate of the ESR signals on several parameters after lyophilization. This may contribute to clarify some of the unkowns.

Materials and Methods

Aqueous solutions of different concentration of ascorbic acid (Merck, Darmstadt) were mixed with ACD-stabilized human blood or Sephadex G 15, respectively. The samples were lyophilized without additional cooling and thereafter exposed to air. ESR measurements were performed with conventional Varian spectrometers working in the X-band (9.4 GHz) and Q-band (34.7 GHz). Modulation frequency was 100 kHz, modulation amplitude 0.2 mT, and microwave power 1 mW. Moisture treatment of the samples was accomplished by storing the lyophilized substance in a vessel containing saturated water vapor at 22 °C. The water content of the samples was not determined, however, the moisture content was so small that no significant change in frequency or Q-value of the cavity could be observed.

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Results and Discussion

Three systems have been investigated after lyophilization: human blood and mixtures of ascorbic acid with human blood and with the inert synthetic material Sephadex, respectively. The results are summarized in Table I.

The ESR spectrum of the ascorbic acid mixtures after lyophilization and exposure to air is shown in Fig. 1. It is an asymmetrical singlet, which grows up to its maximum value in air at room temperature usually after a few hours, and which can be understood as an adsorbed and immobilized ascorbyl radical [2, 6]. The signal intensity of the ascorbic acid mixtures with blood is more than one order of magnitude higher than of the mixture with Sephadex, documenting the strong dependence of the signal intensity on the matrix.

The spectrum at Q-band frequencies shows that the asymmetry, which can be seen at X-band frequencies is not due to a superposition of signals, but to g-value anisotropy. The hight of the signal exceeds the value of the radical spectrum which may be seen in solution by several orders of magnitude. This can be explained by the oxidation of ascorbic acid concomitantly with the adsorption of the ascorbyl radical on an unknown matrix: ascorbic acid $\rightleftarrows$ ascorbyl radical $\rightleftarrows$ adsorbed ascorbyl radical. The free ascorbyl radical, a doublet at $g = 2.005$ with a hyperfine splitting of $a_H = 0.18$ mT can also be recorded after lyophilization. It can, however, only be observed at measuring temperatures higher than about 40 °C and after the reduction of the anisotropic singlet for example by means of moisture treatment (Fig. 2a, b; Table I).

Table I. ESR results of lyophilized samples.

	Blood	Blood/ascorbic acid mixture	Sephadex/ascorbic acid mixture
signal characteristics after lyophilization and exposure to air	nearly symmetrical singlet R_s (low intensity)	anisotropic singlet R_a	
		$g_\perp = 2.0065\ (\pm 0.0002)$ $g_\parallel = 2.0022\ (\pm 0.0002)$ (high intensity)	$\Delta H_{max}(X) = 0.8$ mt $\Delta H_{max}(Q) = 3$ mT (Fig. 1) (low intensity)
... subsequent moisture treatment	slow disappearance of R_s	ascorbyl radical doublet	
		appearance of new signals ($g = 1.999$)	
... subsequent storage at 40 °C	slow increase of R_s $g = 2.005$ $\Delta H_{max}(X) = 0.9$ mT $\Delta H_{max}(Q) = 2.5$ mT (high intensity)	fast increase of R_a signal is finally superposition of R_a and R_s (Fig. 2c, d) (high intensity)	no change of the signal properties

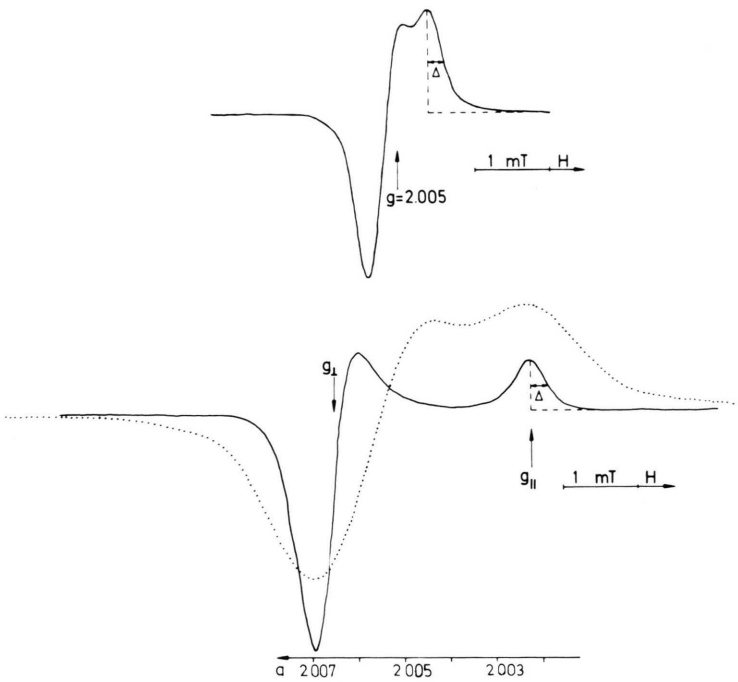

Fig. 1. ESR spectra of a lyophilized mixture of blood and ascorbic acid (100 mM), room temperature measurements. Upper drawing: X-band spectrum. Lower drawing: Q-band spectrum. The dotted curve is a superimposed X-band spectrum in a magnetic field scale extended by a factor of about the frequency ratio $\nu(Q)/\nu(X)$.

At room temperature it can no longer be detected probably due to line broadening which may be caused also by immobilization of the radical. This corresponds to the known property of the ascorbyl radical in certain tissues, where it can be measured in wet tissues, but not in the frozen state [9, 10].

To answer the questions on the relation between the ESR signal after lyophilization and the metabolic state of a tissue before this treatment it is among other informations mandatory to know whether other radicals are covered by the usually dominating anisotropic singlet and what their fate is after lyophilization. Different methods are available: saturation measurements, measurement at higher frequencies, computer simulation and controlled variation of radical composition. The experiments indicated, that saturation measurements cannot be successful due to the inhomogeneous satu-

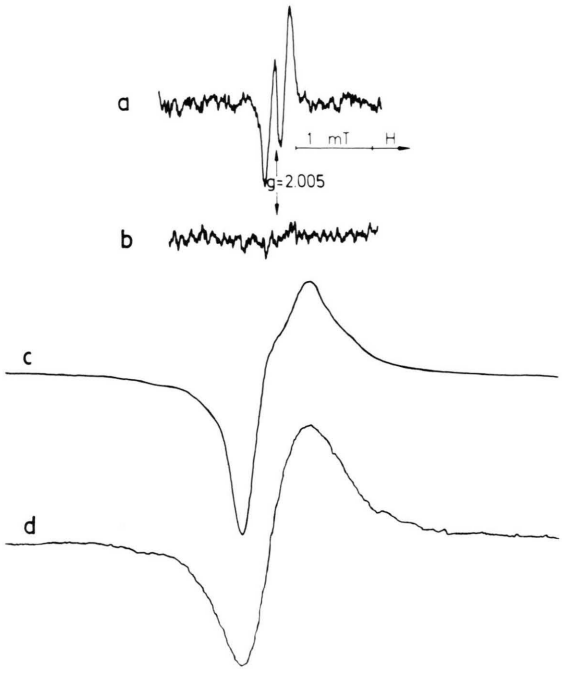

Fig. 2. ESR spectra (X-band) of lyophilized mixtures of ascorbic acid with: a), b) Sephadex, 5 h in moist atmosphere, measuring temperature 50 °C (a) and 21 °C (b). c), d) blood, 30 h in moist atmosphere, 5 d storage at 40 °C, ascorbic acid concentration 50 mM (c) and 1 mM (d).

ration of the anisotropic signal. Moisture treatment [11] of the samples proved not very suitable because of the possible interaction of ascorbic acid or its radical with the matrix in this condition (Table I). Useful methods can be measurements at Q-band frequencies in cases, when relatively narrow signals at about $g = 2.003-2.005$ are buried under the anisotropic singlet (see Fig. 1) and temperature treatment of the samples at about 40 °C.

This treatment leads to an increase of the anisotropic singlet and the nearly symmetrical singlet of the blood as well, however, with different kinetics (Table I). It seems reasonable to attribute this latter signal to semiquinone radicals, which are known to exist before lyophilization [12] and which give symmetrical or slightly asymmetrical singlets after lyophilization and the exposure to air [13, 14]. Treatment with moist air and temperature at 40 °C simulates the storage of lyophilized samples, if they are not kept under vacuum. The same types of signals as shown in Fig. 2c, d can then be detected.

The adsorbing matrix, moisture and temperature, which are parameters with strong influence on the ESR spectra, may already be varied during the lyophilization process. Moisture content and temperature of the samples at the end of the lyophilization process depend of the efficiency of the vacuum pump and the possibility to avoid thawing of local regions of the sample. The latter experimental parameters may cause the lysis of the blood cells [15], an event, which varies the number of potential matrix molecules, the ascorbic acid or semiquinone radicals may interact with. The conditions during the lyophilization process may therefore also influence the radical yield obtained after lyophilization.

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