An Experiment in Electron Paramagnetic Resonance Spectroscopy for Undergraduate Labs in Physical Chemistry or Physical Biochemistry

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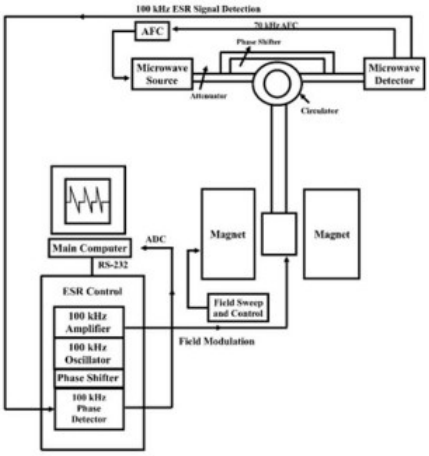
Abstract

We report a biochemically based EPR experiment for undergraduate laboratories that will serve as instruction for learning EPR sample preparation, spectral observation, and spectral analysis. There is a current lack of EPR instructional experiments that present complex spectra in which computational analysis may be required. The biochemical oxidation system of horse radish peroxidase (HRP) activated by H_2O_2 acts on 2,6-di-t-butyl-4-hydroxyanisole (DTBHA) to form phenoxyl radicals. This experiment produces moderately complex spectra of moderately long-lived radicals using relatively small amounts of readily available reactants. Spectrum analysis and simulation is accomplished using software freely available on-line. If desired, EPR studies may be extended to related systems such as the food additives butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) with shorter-lived radicals. If appropriate, molecular orbital calculations at various levels of sophistication may be employed to rationalize EPR results.

Graphical Abstract



Keywords

Upper-Division Undergraduate Laboratories, Biochemistry and Physical Chemistry, Inquiry-based Learning, EPR Spectroscopy

Introduction

Electron paramagnetic resonance (EPR) is a form of magnetic resonance spectroscopy frequently used in the study of electronic structure of free radicals, molecules with unpaired electrons. However, this technique, also known as electron spin resonance (ESR) spectroscopy, is often underrepresented in the undergraduate curriculum. In the *Journal of Chemical Education,* only a handful of experiments in EPR spectroscopy have been published since 2000. (1-5) In determining suitable EPR experiments for an undergraduate laboratory, it is important to design the experimentation around relatively long-lived radicals that are readily available or that can be prepared easily from inexpensive source materials.

Only a few EPR experiments have been reported suitable for the undergraduate laboratory beyond the observation of spectra for simple inorganic systems such as Fremy’s salt (dipotassium peroxylamine disulfonate), (6) inorganic ions such as

Cu(II) and Mn(II), (7) and stable organic radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl) (8) and BDPA (α,γ-Bisdiphenylene-β-phenylallyl) (9) which are commercially available. Some of these have very simple or very complex spectra and do not lend themselves well to illustrating EPR principles beyond instrument operation and spectra recording. An exception is the experiment offered by Beck and Nibler that features EPR observation of benzosemiquinone radicals and molecular orbital calculations at the Huckel Molecular Orbital level. (10) A more complete description of EPR experiments published in the literature and textbooks is given in the Supplemental Information.

EPR Theory:

Because standard textbooks provide detailed background theory, only a brief overview is presented here. (11—13) Electrons all have an intrinsic angular momentum, spin, associated with them. This spin along the z-axis is represented by the quantum number M_S and has the value ±1/2. In chemical bonds, electrons have paired opposite spins hence no net spin angular momentum. When single electrons occupy an orbital, they have a net spin angular momentum and a resulting magnetic moment. By determining the spin of the electron along the z-axis, the magnetic moment of the z-component, μ^Z_E , can also be identified.

$$\mu^z_E = -M_s * g_e * \beta_e = \begin{cases} -(+\frac{1}{2}) g_e \beta_e \ for\ M_s = +\frac{1}{2} \\ -(-\frac{1}{2}) g_e \beta_e \ for\ M_s = -\frac{1}{2} \end{cases} \quad (1)$$

The Lande -g_e factor is a dimensionless number with a value of 2.0023 for a free electron. β_e is the Bohr magneton with value of 9.2733 x 10^{-24} J/T. (13) When there is no external magnetic field, the spin states of the electron have equal energies and are degenerate. By applying a magnetic field, *B,* the electron energies are no longer degenerate and denoted by the equation:

$$E = -\mu^z_E * B = +(M_s g_e \beta_e) * B \quad (2)$$

This change from a degenerate state to non-degenerate states is called the “Zeeman effect.” (13) The energy of M_S= +1/2 is higher in energy than that of M_S= -1/2.

$$\Delta E = E1 - E2 = ge\beta eB \quad (3)$$

The difference in electron energies is the “Zeeman Splitting”, and occurs at a specific resonance frequency, *hu*, for *B* greater than zero: (12)

$$hv = g_e \beta_e B = \Delta E \quad (4)$$

The variable *h* in the equation is Planck’s constant. The frequency and magnetic field are related by a proportionality constant, γ_E , equal to 28.02 MHz/mT. (13)

$$\gamma_E = \frac{v}{h} = \frac{g_e \beta_e}{h} \quad (5)$$

Additional splitting of the Zeeman levels, referred to as “hyperfine splitting,” occurs because the electron spin interacts with nuclear spins of nearby nuclei. (8) For radicals in solution, the hyperfine interaction between electron spin **S** and a nuclear spin **I** is isotropic, and the energy level expression becomes:

$$E = +(M_s g_e \beta_e) * B + a M_s M_I \quad (6)$$

Where a is the hyperfine coupling constant and M_S and M_I are the electron and nuclear spin quantum numbers, respectively. If the electron spin interacts with additional nuclei with spin, additional terms are added to this expression. ESR transition energies may be calculated using the energy expressions for the appropriate energy levels and applying the selection rules ΔM_S =1 and ΔM_I = 0. The relative intensity of a hyperfine line is related to the degeneracy of the spin configurations of the equivalent neighbor nuclei. When observing *n* equivalent nuclei and incorporating the spin quantum number, I, there are 2nI+1 hyperfine lines. If I equals 1/2, for nearby hydrogen nuclei, then there are n+1 hyperfine lines. The intensities of the EPR lines for hydrogen nuclear hyperfine interactions follow Pascal’s Triangle. (13) Figure 1 illustrates both the Zeeman and hyperfine splitting of the energy levels and the resulting EPR spectra for interactions with one and two hydrogen nuclei, respectively. If there are more than one set of equivalent nuclei, the resulting spectra show successive hyperfine splitting

by each set potentially resulting in very complex spectra. A variety of illustrative spectra with qualitative interpretation is given by Bunce. (14) The standard EPR references provide more detailed quantum mechanical treatment of the EPR energy levels and transitions and may be included depending on an instructor's interests. (6, 11, 12)

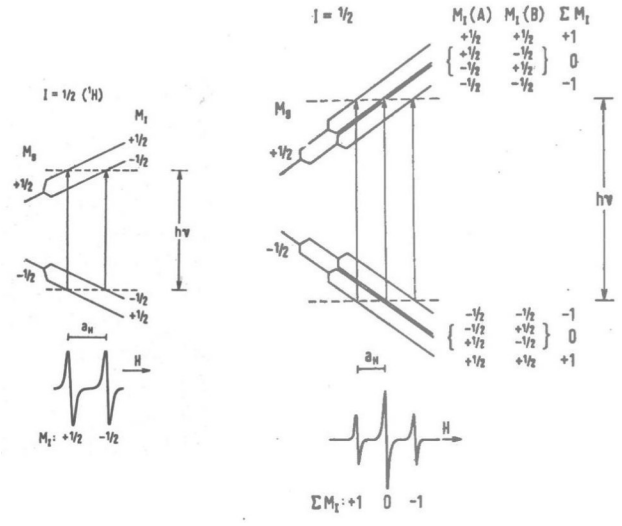


Figure 1: Hyperfine interactions in EPR spectra
(A) Hyperfine interaction with one hydrogen nucleus, (B) Hyperfine interactions with two equivalent hydrogen nuclei. Adapted with permission from Gerson, F.; Huber, W.; *Figures 3.1 and 3.2,* Electron Spin Resonance Spectroscopy of Organic Radicals, Pages 42 and 44, 2006. ISBN: 978-3-527-60524-8. (30)

EPR Instrumentation:

Standard discussions of EPR instrumentation are aimed at practicing spectroscopists and tend to be inaccessible to beginners, (15-17) so this discussion is detailed. As desired, it can be augmented by manufacturers' instruction manuals for specific instruments. See Figure 2 for a generic block-diagram of a typical instrument. The EPR sample is placed in a microwave cavity that is highly resonant (high Q) at a frequency of about 9.4 GHz. The spectrometer sensitivity is directly proportional to the cavity Q-value, hence the use of flat cells or micro-capillaries for aqueous samples to avoid dielectric loss that decreases the cavity Q. The cavity is part of a balanced microwave bridge circuit that includes a microwave source (typically a Gunn diode or, in older instruments, a klystron tube) the frequency of which is "locked" to the cavity frequency by an Automatic Frequency Control (AFC) circuit. The cavity is placed between the poles of an electromagnet. The purpose of the magnet is to align the magnetic moments of the unpaired electrons of the ESR sample into parallel and anti -parallel orientations. Since these two spin states are of different energy, they are populated unequally according to the appropriate Boltzmann distribution. This population difference allows the sample to absorb microwave energy and transfer some spins from the more populated state to the less populated state. We must be careful not to apply too much microwave energy from the microwave source, or we may "saturate" the transition causing the EPR signal to decrease because the populations of the two states become equalized.

When the magnet's field is swept, the magnetic energy levels of the free radicals (determined as the combination of the Zeeman splitting from Bo and the hyperfine coupling of the nuclear and electron spins) change in value and, at certain values of the magnetic field, come into resonance with the available (fixed frequency) microwave energy. These resonances cause imbalances in the microwave bridge, are detected as the EPR spectrum, and are plotted as a function of the field values as it is being swept. The relaxation time for the electrons to return from the excited (less populated) state to the ground (more populated) state is quite rapid compared to the field sweep rate so the line intensities of the spectrum are proportional to the intensities predicted by the n+1 rule for hydrogen hyperfine interactions (provided we avoid "saturation").

The EPR experiment is handicapped by an inherent low signal-to-noise ratio. In spectroscopy in general, the sensitivity of the experiment varies as the frequency of the experimental transitions is raised to the third power. Although we think of 9.4 GHz (~1010 Hz) as a high frequency, it is low compared to IR and UV-VIS frequencies (~1013 and ~1015 Hz, respectively). To improve the S/N we need to reduce the noise because amplifying the signal would also amplify the noise at the same time. The noise reduction technique has three steps. (18) First, the signal is modulated at 100 kHz, which moves it from near dc (~0 Hz) to a region of the noise spectrum that

is relatively noise free. This is accomplished by superimposing a 100 kHz “wobble” on the slowly increasing ESR magnetic field by applying a 100 kHz modulation signal to coils in or around the microwave cavity. Because of this modulation, the resonance microwave absorption during the ESR transitions in turn is modulated at 100 kHz. Second, the signal at the microwave detector of the microwave bridge is passed through a narrow band (“notch”) filter tuned to 100 kHz, amplified, and then demodulated at 100 kHz. This means that only the noise in the detected microwave signal is happening at 100 kHz. Third, the 100 kHz demodulated signal is phase detected at a phase synchronous with the phase of the modulating signal so that any noise that is out of phase is suppressed. The overall improvement in S/N can be on the order of 105 or 106 because of this electronic massaging of the ESR signal. The final output can also be low-pass filtered depending on how fast the experiment is conducted, digitized by an analog-to-digital converter, displayed on a computer monitor and/or saved as a disk file. Because of the modulation-demodulation detection scheme, EPR spectra generally appear as first derivative spectra. Depending on the available software, digitized EPR spectra can be processed additionally to provide smoothing, integration, etc. A more detailed discussion of the S/N problem is given by Coor. (19, 20)

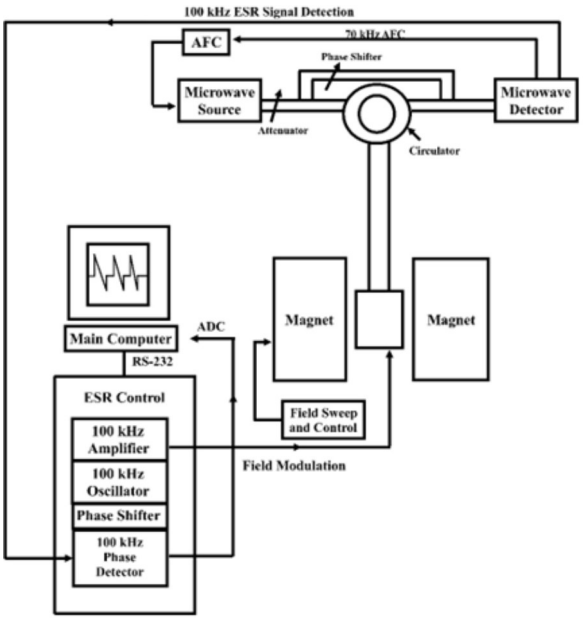


Figure 2: EPR Spectrometer
A generic block diagram of the internal components of an EPR spectrometer. Components identify the critical components of EPR spectrometers.

Biochemical Oxidizing System:

Horseradish peroxidase (HRP), when activated by hydrogen peroxide, is a robust, convenient, commercially available oxidizing system that can accept a wide variety of substrates. (21, 22) The catalytic cycle of HRP is clearly presented and discussed by Dawson, and Figure 3 is adapted from that paper. (23) We have found this to be an excellent reference to introduce students to the topic of peroxidase enzyme catalytic activity.

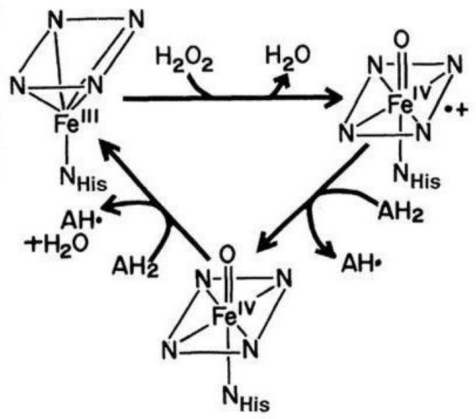


Figure 3: Catalytic cycle of horseradish peroxidase.
The overall charge on the ferric state (top left) and on the compound I (top right) is +1, whereas compound II (bottom center) is neutral. Reproduced, with permission, from Dawson, J. H. Probing Structure-Function Relations in Heme-Containing Oxygenases and Peroxidases. *Science*, 1988, *240*, 433-439.

The top left structure is the resting enzyme. Upon activation by hydrogen peroxide, it is designated as Compound I that is two oxidizing equivalents above the resting state and is symbolized by the ferryl iron and a cation radical assigned to the porphyrin ring system. Upon reacting with a substrate designated by AH_2, Compound I becomes Compound II which is still one oxidizing equivalent above the resting state and can complete another oxidation of another substrate molecule. For students desiring

more extensive information about HRP, detailed structural descriptions of ferryl heme in Compounds I and II have been reported. (24)

In our experiment the substrate is a phenol, specifically either 3,5-di-t-butyl-4-hydroxyanisole (DTBHA) or 2,6-di-t-butyl-*p*-cresol (butylated hydroxytoluene, BHT) and HRP oxidation of the substrate produces the corresponding phenoxyl radical. The phenoxyl radical of DTBHA is moderately long-lived under our conditions, *e.g*., 10 – 30 minutes but that of BHT has a distinctly shorter lifetime of *ca.* 1 – 5 minutes.

Experimental

Procedure:

EPR spectra were measured at room temperature using a JEOL-RE-1X spectrometer operated near a frequency of 9.4 GHz and a magnetic field near 340 mT. The microwave cavity was modulated with a magnetic field of 100 kHz. All chemicals were acquired from Sigma-Aldrich and used as received except that DTBHA and BHT compounds were recrystallized once from EtOH. Incubations were prepared as 5 mL samples that were 50% v/v 100 mM tris pH 7.4 buffer and ethanol from concentrated reagent stock solutions, and as prepared contained 0.42 mM DTBHA, 0.23 μM HRP, and when activated were 1 mM H_2O_2 . Detailed procedures for preparation of the buffer and the reagent stock solutions are in the Supplemental Information.

Data analysis:

The spectra were measured with a LabVIEW 7.1 interface written locally and saved as digitized ASCII files. The spectra were analyzed using the WinSim 2002 software from NIEHS (Research Triangle Park, NC). (25) LabVIEW itself was purchased from National Instruments (Austin, TX). Files were converted from LabVIEW to a WinSim usable format using a conversion program written locally. When performed, the density functional theory (DFT) molecular orbital spin density calculations were completed using GaussView and Gaussian ‘03 software purchased from Cyberchem (Gainesville, FL). (26)

The WinSim software package is a free download and provides a rich suite of spectra processing features including baseline correction and centering of spectra, autocorrelation determination of possible hyperfine coupling constants, simulation of trial spectra, optimization of trial spectra to fit experimentally-observed spectra by variation of hyperfine coupling constants and line widths, display of optimized spectra, calculation and display of the residual difference between observed and simulated spectra, and reporting of a numerical correlation parameter for the goodness of fit. Additionally, WinSim can simulate multiple EPR spectra corresponding to different radical species present in the same sample and optimize the concentrations of those species. The computer program supporting WinSim is described by Duling (27, 28) and the overall WinSim package is described at the NIEHS PEST (Public Electron Paramagnetic Software Tools) website. (25)

Hazards and Safety Precautions

This project will use basic laboratory equipment and basic safety precautions: goggles, gloves, and proper clothes should always be worn. HRP is not hazardous according to OSHA criteria. DTBHA is harmful if swallowed and can cause serious skin, eye, and respiratory irritation. B TBHA is also a suspected carcinogen. DTBHA release to aquatic environments should be prevented, but otherwise the substance is not classified as hazardous in a laboratory setting. (14) H_2O_2 is harmful if swallowed and can cause serious eye damage. The release of H_2O_2 into aquatic environments should be prevented.

Results and Discussion

Figure 5 illustrates the EPR spectrum that is observed for a sample of DTBHA oxidized to its phenoxyl radical by HRP activated by H_2O_2 and placed into a 50 μL micropipette. The simulated spectrum used values of 0.166 mT for the three methoxy hydrogen atoms and 0.074 mT for the two *meta*- phenoxyl ring hydrogen atoms. Blocking of the *ortho*- phenoxyl ring positions by the *t*-butyl substituents results in the spectrum being characterized by only those two kinds of hydrogen couplings. The correlation constant for this simulation is 0.997. These HFCC parameters are quite close to the published values given in the caption to Figure 6.

Those values were obtained without the benefit of the WinSim optimization.

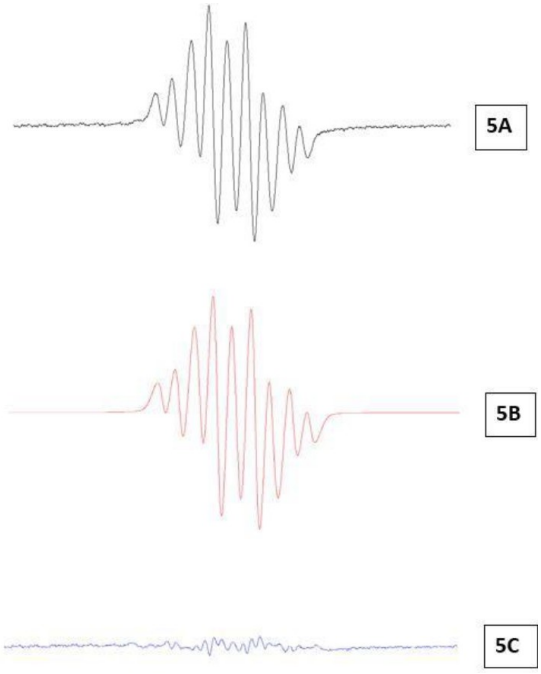


Figure 5: DTBHA Phenoxyl Radical EPR Spectrum and Analysis

Complete system (A) experimental EPR spectrum of the DTBHA phenoxyl radical identified in (29) alongside (B) simulated EPR spectrum of a DTBHA phenoxyl radical, and (C) the residual difference plot of the experimental and simulated spectra.

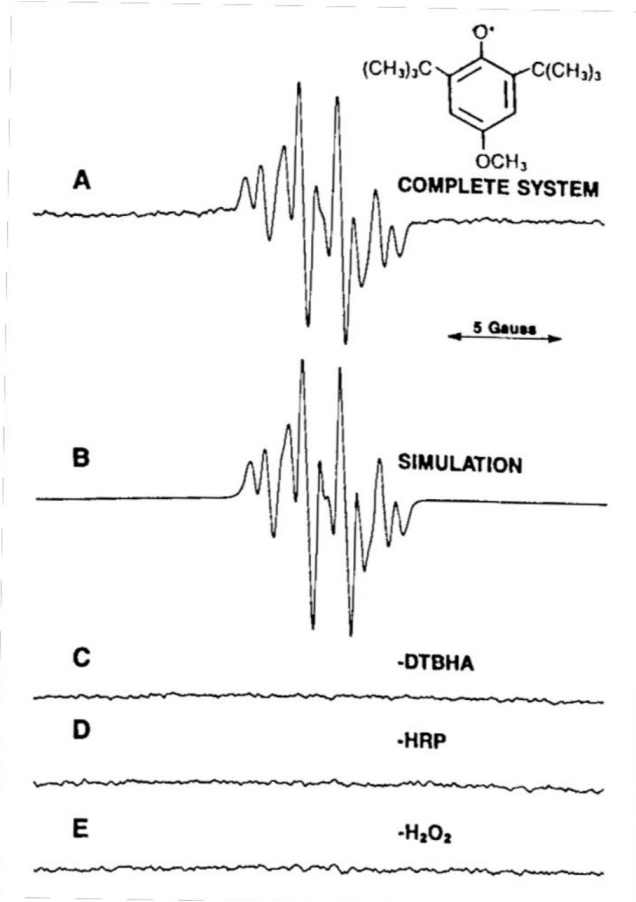


Figure 6: EPR spectrum of DTBHA Phenoxyl Radical

Complete system (A) of 1.06mM DTBHA, 1.0 mM H_2O_2, and 10 µg/mL horseradish peroxidase (HRP), in Tris-HCl buffer, pH 8.0; incubation 12.5% (v/v) ethanol and bubbled with N2 before aspiration into the EPR f lat cell. Instrument conditions: microwave power, 20 mW; sweep rate, 25 G/200 s; modulation amplitude, 0.25 G; time constant, 1.0 s; gain, 5.0 x 105. (B) Computer simulation of DTBHA radical EPR spectrum (aHOCH3 =1.70 G (3H), aH3,5 = 0.63 G (2H)). (C) Same as A, but without DTBHA. (D) Same as A, but without HRP. (E) Same as A, but without H_2O_2. Reproduced, with permission, from Valoti, M.; Sipe, H.J.; Sgaragli, G.; Mason, R.P. Free Radical Intermediates during Peroxidase Oxidation of 2-t-Butyl-4-methoxyphenol, 2,6-di-t-butyl-4-methylphenol, and Related Phenol Compounds. *Arch. Biochem. Biophys.* 1989, *269*, 423-432. (29)

The proposed experiment allows for an effective teaching standard of EPR spectroscopy for

undergraduate physical chemistry and biochemistry students.

The expected result of this project is to generate an experimental spectrum that is suitable for undergraduate analysis and demonstrates the fundamental principles of EPR spectroscopy. The use of this experiment is appropriate for use in undergraduate physical chemistry and physical biochemistry laboratories by virtue of the simplicity of the experiment and the resulting spectra.

Conclusion

The biochemical oxidation of the DTBHA and BHT compounds using the HRP/H2O2 system produces EPR spectra that offer significant instructional benefit for undergraduate physical chemistry or physical biochemistry laboratories. This EPR experiment presents novel opportunities to utilize EPR spectrometers to complete interesting demonstrations and instructional experiments in undergraduate physical chemistry or physical biochemistry laboratories.

Associated Content

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.XXXXXXX.

- Sample Lab handout for EPR Spectroscopy of Horse-Radish Peroxidase Oxidation of 3,5-di-t-butyl-4-hydroxyanisole

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Acknowledgements

We wish to acknowledge Hampden-Sydney College, the Massey Foundation, and the National Science Foundation's ILI Program for the funding for the EPR spectrometer used in this study.

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