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Unprecedented stable aqueous semiquinone methide radical formation interferes with adsorptive cathodic stripping voltammetry of cobalt methyl thymol blue

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Abstract

A putatively highly sensitive and selective method for the determination of cobalt in aqueous samples by catalytic adsorptive cathodic stripping voltammetry using methyl thymol blue (MTB) as the ligand has been documented [A. Safavi, E. Shams, Talanta 51 (2000) 1117] and its underlying mechanism has been briefly explored [A. Safavi, E. Shams, Electroanalysis 14 (2002) 708]. In an attempt to adapt the method for application in metalloprotein analysis we obtained erratic results, which were traced down to the redox non-innocence of the free ligand in the potential range prescribed for the metal analysis. On the hanging mercury drop electrode free methyl thymol blue is reversibly one-electron reduced to the semiquinone form with $E_{m,7.0} = -482$ mV versus NHE at 22 °C, and the radical is subsequently quasi-reversibly one-electron reduced to the quinol form with $E_{m,7} \approx -0.9$ V. This observation invalidates the use of MTB in electrochemical analysis of metal ions. This is also the first observation ever of a stable quinone methide radical in aqueous solution.

Keywords: Stripping voltammetry; Cobalt determination; Methyl thymol blue; Quinone methide

1. Introduction

Catalytic adsorptive cathodic stripping voltammetry (ACSV) is a rapidly emerging analytical technique for the trace determination of metal ions in aqueous solutions, in which the cation is complexated by a ligand with adsorptive tendency towards a mercury electrode and an additional weakly ligating oxoanion as oxidator recycles the reduced metal [1]. A recently described variant uses the presumably redox-inert 3,3′-bis(*N*,*N*′-di[carboxymethyl]aminomethyl) thymolsulfonphtalein, or methyl thymol blue (MTB, Fig. 1), as the adsorptive ligand with nitrite as the oxidant for the determination of cobalt(II) with reported high selectivity versus a background of nickel(II) [2,3].

ACSV-based determinations are typically intended for ultra-trace measurements in, e.g., natural waters. As part of a long-term proteomics research effort on metalloproteins we attempt to modify these described procedures for our determinations of metal ions as cofactors in proteins, i.e. at significantly higher concentrations, however, versus a massive organic background of (bio)polymer. An example is the determination of molybdenum and/or tungsten in complex enzymes [4]. In an attempt to adopt the cobalt-MTB method for application in metalloprotein studies we found the originally reported voltammetry [2,3] not well reproducible, and this was traced down to electrochemical reduction of the free ligand MTB. This observation invalidates MTB as a useful ligand in ACSV-based metal ion determination; however, it is also the first observation of reversible aqueous redox chemistry of a quinone methide.

Quinones are ubiquitously functional in biology (hence the name ubiquinone) e.g., as relay centers of electron and proton gradients in respiratory chains. Their redox chem-

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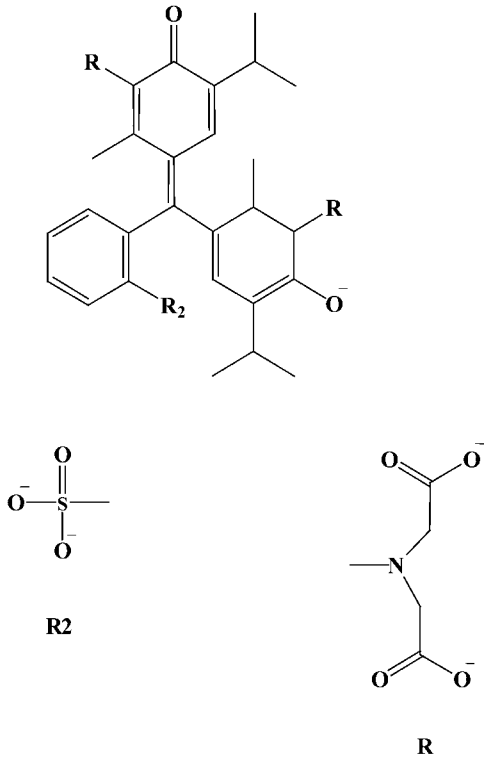

Fig. 1. Methyl thymol blue: a quinone methide.

istry has been very extensively documented both in aqueous and in non-aqueous media [5–7]. Quinone methides increasingly appear to be of comparable biochemical importance where they have been implicated as intermediates in a myriad of processes, notably in the synthesis and breakdown of biopolymers, e.g., skin melanin [8], wood lignin [9], insect cuticles [10], in the covalent flavinylation of enzymes [11], in catabolism of food ingredients [12], and for their toxicological properties against normal and cancerous cells [13], e.g., through DNA alkylation [14]. However, in comparison to quinones our knowledge of the redox chemistry of quinone methides is significantly more limited [15–18].

The rich redox chemistry and biochemistry of quinones [5–7] is conceptually simple: in aqueous solution and in biological fluids a quinone is a two-electron acceptor, whereas in apolar solvent and in biological membranes the semiquinone radical is stabilized usually with well separated reduction potentials each positive to that of the normal hydrogen electrode (NHE). For quinone methides (Fig. 2) in apolar medium the

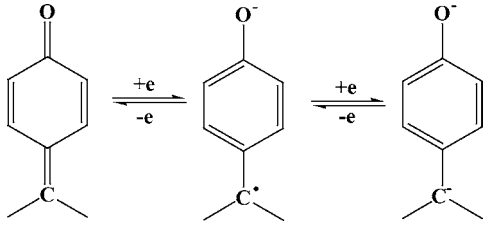

Fig. 2. One-electron reductions of a quinone methide.

two E_m's are typically 1–2 V more negative than for quinones [15]. Remarkably, aqueous redox chemistry of quinone methides has never been reported.

2. Experimental

2.1. Reagents

3,3′-bis(*N*,*N*′-di[carboxymethyl]aminomethyl)thymolsulfon-phtalein, or methyl thymol blue, was obtained from Sigma-Aldrich and was used as received. Good's buffers were obtained from Sigma (Mes, Hepes, Epps), MP Biomedicals (Tris), Serva (Ches), and Fluka (Caps), and were used to make a pH solution range of approximately constant ionic strength as 500 mM KCl plus 50 mM buffer, namely Mes, pH 6.0 and 6.5, Hepes, pH 7.0, Epps, pH 7.5 and 8.0, Tris, pH 7.5, 8.0, 8.5, Ches, pH 8.5, 9.0, 9.5, and Caps, pH 10, 10.5, 11.0. Sodium dithionite was from Fluka; fresh anaerobic solutions under argon were quantitated using $\varepsilon_{315} = 8\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$ according to [19].

2.2. Electrochemistry

Cyclic voltammetry was measured with an Autolab station (ECO chemie, The Netherlands) consisting of a PSTAT10 digital potentiostat equipped with an ECD module (low current amplifier) and using GPES 4.8 software. The electrochemical cell was the HMDE 663 VA Stand (Metrohm Switzerland) with a hanging mercury drop working electrode, a glassy carbon rod counter electrode, and a double-junction Ag/AgCl (3 M KCl) reference electrode. For experiments with acid-activated glassy carbon as working electrode the previously described microcell was used [20].

The pH dependence of the reduction potential (or midpoint potential) E_m was analyzed using the general equation (cf p. 147 of [5]),

$$E_m = E_{m,0} + \left(\frac{RT}{nF}\right) \ln\left(\frac{X_{red}}{X_{ox}}\right)$$

in which,

$$X_i = [H^+]^a + K_{i1}[H^+]^{a-1} + K_{i1}K_{i2}[H^+]^{a-2} + \cdots + (K_{i1}K_{i2}\ldots K_{in})$$

K_{red} and K_{ox} are proton dissociation constants of the reduced and oxidized compound, respectively, and the numerical subscript defines the order of subsequent K's from low to high pH. For the case at hand (three K's; see below) this becomes

$$X_i = [H^+]^3 + K_{i1}[H^+]^2 + K_{i1}K_{i2}[H^+] + K_{i1}K_{i2}K_{i3}$$

and it is understood that K_{red3}, K_{ox1}, and $E_{m,0}$ are in the present work ($6 \le pH \le 11$) dummy parameters because K_{red3} refers to pH > 11 and the other two parameters refer to pH < 6.

2.3. Optical spectroscopy

Absorption spectra were recorded with a Hewlett-Packard 8452A diode array spectrophotometer with a 1-cm path length anaerobic cuvette. The rate constant for the reaction of MTB with sodium dithionite was calculated from the equation for an irreversible second-order reaction,

$$k = \tau_a^{-1}(b-a)^{-1} \ln\left[\frac{(2b-a)}{b}\right]$$

in which b is the initial dithionite concentration, a is the initial MTB concentration, and τ_a is the experimentally determined half-life for the reduction of MTB.

2.4. EPR spectroscopy

Data were recorded on a Bruker 200 D EPR spectrometer equipped with a home-built cryogenic flow system for He or N_2 with temperature measurement just below the sample using a 5 kΩ carbon composition resistor as thermometer. The g-values of the MTB radical were determined with respect to the spectrum of the strong pitch sample ($g = 2.0028$) using a Hewlett-Packard 5245L electronic counter equipped with a 5255A frequency converter. The MTB radical spin concentration was determined by comparison of the spectrum second integral with that of the external standard 10.00 mM $CuSO_4$, 10 mM HCl, 2 M $NaClO_4$. The radical spectrum was simulated [21] assuming a Gaussian line shape symmetrical in field space from unresolved superhyperfine structure, i.e. using the spin Hamiltonian,

$$H = g_{\parallel}\beta B_z S_z + g_{\perp}(B_x S_x + B_y S_y) + W_{\parallel}\beta B_z S_z + W_{\perp}(B_x S_x + B_y S_y)$$

in which W_i is a line width component, namely, a standard deviation of a Gaussian distribution in field values. The final fit was obtained with a Simplex minimizing routine.

3. Results and discussion

3.1. Electrochemistry of the radical

When attempting to reproduce the determination of cobalt as its MTB complex as described in the literature [2,3] we obtained significant responses even in the absence of metal. MTB at concentrations of 5–100 μM in buffer solution exhibits a response in cyclic voltammetry on the hanging mercury drop electrode (HMDE) with characteristics of a reversible one-electron reduction to the semiquinone methide form as illustrated in the right side of Fig. 3.

In voltammograms over a limited potential scan (−0.65 to −0.90 V) the cathodic-to-anodic peak potential separation, ΔE, is approximately 60 mV independent of the potential scan rate at least up to $v = 0.6\ \mathrm{V\ s^{-1}}$. The ratio of cathodic-to-anodic peak currents is approximately unity, and the peak

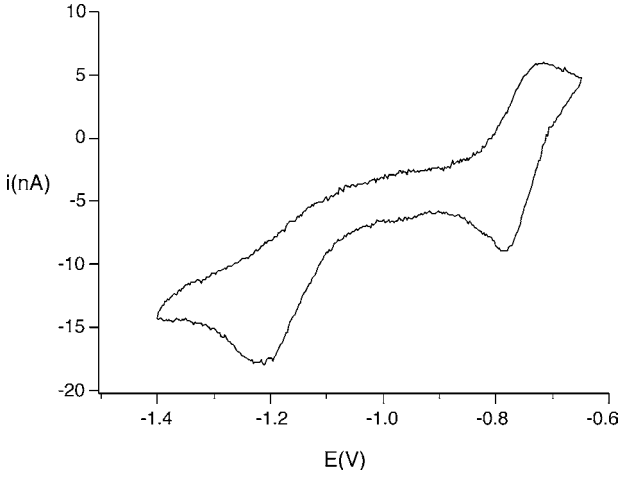

Fig. 3. An example of a cyclic voltammogram of methyl thymol blue on the hanging mercury drop electrode. The first reduction wave, reflecting the formation of semiquinone methide radical, is reversible and stable in time when the scan is reversed at −0.9 V. The second reduction wave, from the formation of the quinol methide, is quasi-reversible and of limited stability. The cell contained 40 μM MTB in 500 mM KCl and 50 mM Ches buffer, pH 9.0. The potential scan rate was 60 mV/s. The figure shows the sixth scan after initiation.

current amplitude, i_p, increases linear with the square root of the potential scan rate. The response is stable for prolonged periods of time, typically up to at least an hour at ambient temperature. An intrinsic property of the HMDE, when compared to, e.g., carbon electrodes, is low amplitude of non-Faradaic background current affording well developed voltammograms at low concentration of electroactive species. Using higher concentrations of MTB similar responses, be it with much higher non-Faradaic background, were obtained on nitric acid-activated glassy carbon (not shown).

At approximately 400 mV more negative potential a second wave is observed indicative of further reduction of the quinone methide radical to the two-electron reduced quinol methide, as illustrated in the left side of Fig. 3, however, this response is quasi-reversible and not stable in time. The peak separation increases with potential scan rate, and the average potential and the peak currents are not well reproducible in repeated scans, and eventually disappear, suggesting production of the dianion which is apparently not chemically stable under the conditions employed.

3.2. Dependence on pH of the reduction potential

MTB (i.e. the oxidized quinone methide) has been previously studied extensively with optical spectroscopy with a view to its application in colorimetric metal determination. Yoshima et al. determined six pK_a values of uncomplexed MTB in the pH range from 0 to 14, and they also proposed an assignment to the carboxylate and sulfonate side groups (cf Fig. 1) based on comparison with the pH dependent spectra of metal complexed MTB [22]. We have studied the electrochemistry of MTB over the potentially biochemically relevant pH range of 6–11, and this range encompasses two

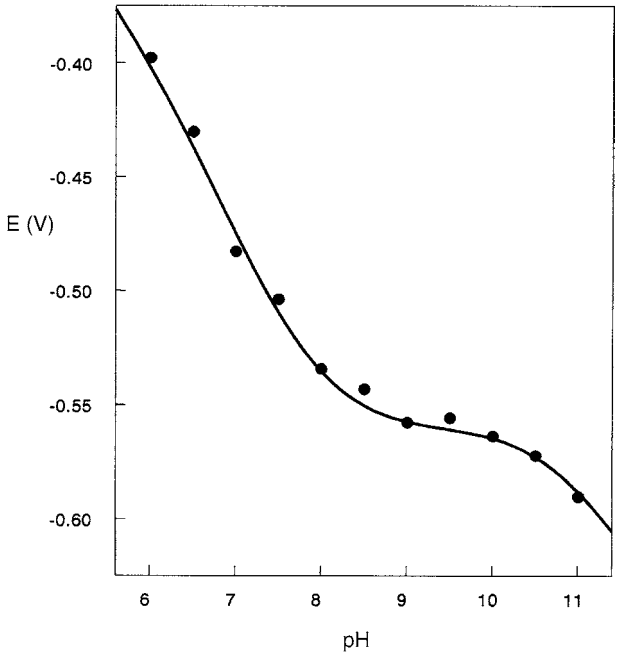

Fig. 4. The reduction potential for the quinone methide/semiquinone methide transition of methylthymol blue as a function of the pH at 22 °C. The midpoint potentials were determined from stable voltammograms over a limited potential scan down to −0.9 V. The solid line is a fit using pK_a-values of 6.6 and 10.7 plus one undermined value ≪ 6 for oxidized MTB and pK_a-values of 7.2 and 8.2 for one-electron reduced MTB.

previously identified carboxylato pK_a values of 6.9 and 11.1, therefore, the reduction potentials can be anticipated to exhibit pH dependence.

Because of the instability of the second reduction step quantitative data have been obtained only for the reduction from quinone to semiquinone, and these are presented in Fig. 4. The reduction potential for the first step is not significantly affected by changes in buffer concentration (twofold), changes in buffer composition (e.g., Epps ↔ Tris), or changes in MTB concentration (5–100 μM).

Initial scans over wider potential range indicate that the E_m for the second step is always approximately 0.3–0.5 V more negative than the E_m of the first step over the whole pH range 6–11 (not shown). The dependence of the first E_m on pH is very strong at low pH with a negative slope well over 59 mV per pH unit, which indicates that more than one proton is bound upon one-electron reduction and that this part of the curve is determined by two pK_a values of the oxidized MTB. At higher pH values a plateau is followed by another slope, therefore, reproduction of the data requires in total three pK_a values for oxidized MTB and two pK_a values for the semiquinone form, where one value of the oxidized form is undetermined because the graph begins with a finite slope (cf Section 2). The fit shown in Fig. 4 gives for oxidized MTB pK_a = 6.6 and 10.7, and for semiquinone MTB pK_a = 7.2 and 8.2. The values for oxidized MTB compare reasonably well with the fourth and fifth pK_a = 6.9 and 11.1 determined before in optical titrations [22].

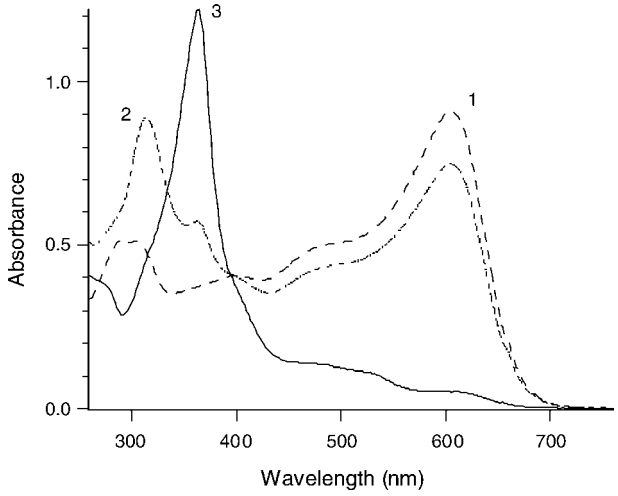

Fig. 5. UV–visible spectra of methyl thymol blue in the course of its reduction by sodium dithionite to the semiquinone methide form. The broken line (1) is oxidized MTB at $t = 0$ (no reductant present); directly after mixing with dithionite the dotted line (2) is obtained, which is a mixture of quinone methide (peak at 604 nm), semiquinone methide (peak at 364 nm), and dithionite (peak at 315 nm); the solid line (3) is from the radical obtained after 1000 s incubation with reductant. The initial reaction mixture contained 42 μM MTB, 120 μM $Na_2S_2O_4$, and 50 mM Ches buffer, pH 9.0.

We note that for pH ≤ 9 the E_m is below that of the hydrogen electrode couple H_2/H^+, and that for pH ≥ 7 the E_m is well below that of the in biochemistry popular low-potential mediator methyl viologen which has a pH-independent $E_m = -440$ mV for the first reduction step [5].

3.3. Optically monitored kinetics

The low potential reductant sodium dithionite, $Na_2S_2O_4$ is widely used in redox studies in aqueous solutions especially of biological systems. Because the actual reductant is the $SO_2^{\bullet -}$ radical, the redox potential of dithionite is a complex function of its concentration and of pH [23]. However, for micromolar concentrations the effective reduction potential is predicted to be approximately in between the two subsequent E_m's of MTB over the pH range employed here. For example at pH 9 we have E_m (MTB ox/semi) = −557 mV, E_m (100 μM dithionite) ≈ −0.72 V, and E_m (MTB semi/red) ≈ −1.0 V. Thus, dithionite should be an approximately stoichiometric reductant of MTB to the semiquinone methide.

In Fig. 5, we show optical spectra of oxidized MTB, after brief incubation with dithionite, and after prolonged incubation with dithionite. The spectrum of dithionite has an absorption band at 315 nm. In the final spectrum a pronounced absorption is found at 364 nm and the intense blue color of oxidized MTB has been replaced by a faint pink color (dithionite is essentially colorless in the visible range).

The three spectra define useful wavelengths to monitor the kinetics of MTB reduction, namely, 604 nm (predominantly oxidized MTB), 364 nm (mainly semiquinone methide), and 315 nm (mainly dithionite).

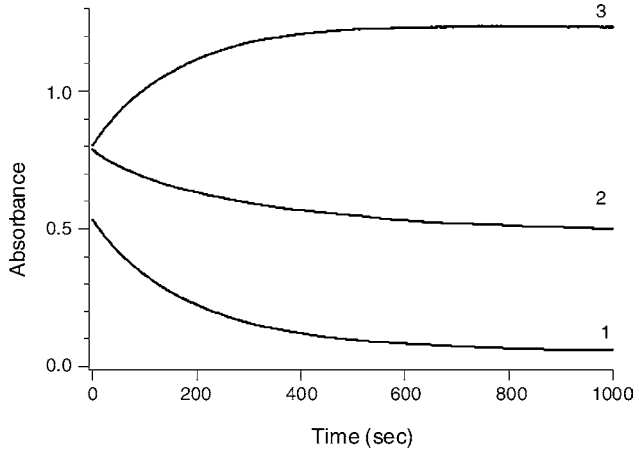

Fig. 6. Optically monitored time course of the reduction of methyl thymol blue by sodium dithionite. The lower trace 1 (604 nm) is the reduction of the blue MTB; the middle trace 2 (315 nm) is dithionite oxidation; the upper trace 3 (364 nm) is formation of the faintly pink semiquinone methide radical. Conditions were as in Fig. 5.

In Fig. 6, the time development of the absorbance at the monitored wavelengths is given for the first 1000 s illustrating the formation of a stable semiquinone methide. On significantly longer time scales spectral changes occur that may be related to radical instability; we have not further investigated these changes. When the change in absorption (increase) at 364 nm and the change in absorption (decrease) at 604 nm are plotted on a scale from 0 to 100%, the curves are nearly mirror images with a cross over close to 50% at $t = 117$ s (not shown). Using this number in the half-life expression for an irreversible second order reaction gives rate constant, $k \approx 55\ M^{-1}\ s^{-1}$.

3.4. EPR spectroscopy of the radical

From the optically monitored time course and the literature values of the dithionite reduction potential it follows that MTB is largely convertible to the semiquinone methide over the pH range of 6–11 by incubation of sub-millimolar concentrations of MTB with slight-to-moderate excess of dithionite, therefore, the radical can be studied by quantitative EPR spectroscopy.

In Fig. 7, we present the EPR spectrum under non-saturating conditions of a frozen solution of 40 μM MTB after 10 min incubation with excess dithionite under argon. The amplitude of this spectrum is linear in the concentration at least up to 250 μM MTB; down to at least $T = 20$ K the spectral shape is independent of temperature and the amplitude is inversely proportional to the temperature following Curie's law (not shown). Quantitation of the spectrum (i.e. double integration) with respect to that of a Cu(II) standard gives a spin concentration of 41 μM.

The shape of the spectrum is rather featureless which is quite common for frozen aqueous solutions of organic radicals where hyperfine interactions are frequently not resolved

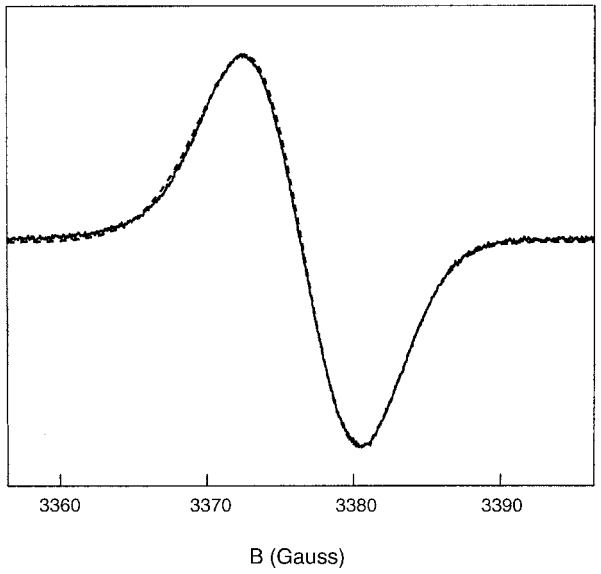

Fig. 7. EPR spectrum of the semiquinone methide radical resulting from reduction of methyl thymol blue with sodium dithionite. MTB, 40 μM, in 50 mM Ches buffer, pH 9.0, was incubated with 2 mM $Na_2S_2O_4$ for 10 min at ambient temperature and then frozen in liquid nitrogen. EPR conditions were: microwave frequency, 9428.4 MHz; microwave power, 8 μW; modulation frequency, 100 kHz; modulation amplitude, 1.25 Gauss, temperature, 115 K. The spectrum (solid line) is an average of 10 scans. The simulation (dotted line) assumes $g_{||} = 2.0042$, $g_{\perp} = 2.0022$, and line widths $W_{||} = 5.94$ Gauss, and $W_{\perp} = 7.76$ Gauss.

presumably due to *g*-strain broadening. However, simulation of the spectrum as a single isotropic line gives some misfit, which indicates the presence of anisotropy. An optimized simulation (dotted trace in Fig. 7) on the basis of an axial *g*-matrix with $g_{||} = 2.0042$ and $g_{\perp} = 2.0022$ essentially fits the experimental spectrum.

4. Conclusions

Adsorptive stripping voltammetry is frequently used for the determination of trace elements. In recent years detailed mechanistic proposals have been put forth, for example, for the combination of the complexing adsorbent dimethylglyoxime and the metal ions Ni(II) and Co(II) in aqueous solution [24–26]. From these studies it has become clear that the ligand is not innocent in the sense that it participates itself directly in the redox chemistry. MTB is another potentially interesting ligand for Co(II) determination [2], however, the mechanism of the Co-MTB stripping voltammetry is not understood, and a direct involvement of the MTB ligand in the redox chemistry has not yet been considered [3]. We have now found that MTB exhibits direct electrochemistry under conditions that are reminiscent of those used in the adsorptive stripping voltammetry of the Co(II) complex, and this suggests that the process for Co-MTB might be mechanistically similar to that proposed [1,24–26] for, e.g., Co(II)-dimethylglyoxime. However, the added com-

plexity that the ligand is electroactive also in its uncomplexed form disqualifies MTB as a practical ligand in these determinations.

The electrochemical activity of MTB is a surprise because MTB is a quinone methide, and for this class of compounds no aqueous redox chemistry is known. Especially intriguing is the observation of two subsequent single-electron reductions when compared to the single-step two-electron reduction of quinones in aqueous solution [5–7]. The semiquinone methide radical is remarkably stable and can be generated quantitatively in reduction chemistry in homogeneous solution.

Numerous biological redox centers have reduction potentials close to, or significantly below the formal potential of solvent reduction, i.e. the H_2/H^+ couple [27–29]. For their characterization there is a lack of practical low-potential redox mediators, e.g., in redox titrations with solid-state indicating electrodes, or in enzyme activity determinations with artificial electron donors. Methyl viologen semiquinone anion radial, with $E_m = -440\,mV$ (independent of pH), is the lowest-potential mediator widely used, where mediators of lower potential, e.g. viologen derivatives, suffer from low stability and or low solubility in aqueous solution. The low potential stable aqueous MTB quinone methide radical is a potential candidate to fill this gap.

Quinone methides have a much wider biological significance because they are, for example, considered to be intermediates in covalent flavinylation of enzymes, to mediate toxicity of quinone antitumor drugs, to be intermediates in the synthesis and the breakdown of various biopolymers [8–14]. The aqueous redox chemistry identified in the present paper may well bear relevance to future studies into the biochemistry of quinone methides.

Acknowledgements

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