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Ascorbate–quinone interactions: Electrochemical, free radical, and cytotoxic properties

(redox potential/semiquinone radical/cancer drug/electron transfer/ascites)

RONALD PETHIG*, PETER R. C. GASCOYNE, JANE A. MCLAUGHLIN, AND ALBERT SZENT-GYÖRGYI

Laboratory of the National Foundation for Cancer Research at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Contributed by Albert Szent-Györgyi, October 12, 1982

ABSTRACT **Standard midpoint potentials have been determined for *p*-benzoquinone, methoxy-*p*-benzoquinone and 2,3-, 2,5-, and 2,6-dimethoxy-*p*-benzoquinones in aqueous solution. ESR studies have been made of the ascorbate and semiquinone radicals produced when these quinones interact with sodium ascorbate. Direct correlations are found between the electrochemical potentials, generated semiquinone lifetimes, and cytotoxic action in Ehrlich ascites-bearing mice.**

This work follows from earlier considerations of the oxidation–reduction and cytotoxic properties of some methoxyquinones (1) and of charge-transfer reactions and electronic delocalization effects in biological systems (2–4). The compounds of interest in these particular studies have been the methoxy-substituted *p*-quinones, two of which (methoxy- and 2,6-dimethoxy-*p*-benzoquinone) occur naturally in wheat germ (5).

Bachur *et al.* (6) have suggested that the cytotoxic action of quinone anticancer drugs is mediated through their free-radical metabolites. An important first step in such activity is the production of relatively stable semiquinone free radicals. Radicals result from one-electron rather than two-electron redox reactions and the radical concentration and rate of production should depend on the difference between the electrochemical potentials of the electron donor and acceptor molecules involved. In this work, electrochemical and ESR measurements have been made to determine the correlation between the cytotoxic activity of several quinone/ascorbate mixtures and their ability to produce stable semiquinone radicals.

MATERIALS AND METHODS

Pure samples of *p*-benzoquinone, methoxy-*p*-benzoquinone, and the three dimethoxy- (2,3-, 2,5-, 2,6-) *p*-benzoquinones, as well as the corresponding hydroquinones, were supplied by G. Fodor (West Virginia University, Morgantown).

The redox potentials of the various quinone–hydroquinone couples were determined at 25°C by using electrodes of bright platinum wire (24 gauge). These were subjected to a nonoxidizing cleaning procedure (7) by placing them for 10 min in boiling 10% sodium bisulfite and then washing in distilled water. The reference half-cell consisted of a saturated solution of quinhydrone (Eastman Kodak) in nitrogen-saturated aqueous 0.01 M HCl/0.09 M KCl, and a 0.5 M KCl/5% agar salt bridge was used as the connection to the test half-cell. The combined electrode and salt-bridge junction errors were measured to be less than 0.2 mV and, when calibrated against a platinized platinum/hydrogen electrode, the standard potential (pH 0) of the quinhydrone reference electrode was determined as 700 ± 1 mV, in good agreement with the values 699.58 mV and 699.92 mV obtained by earlier workers (8, 9). The electrode potentials were measured by using a Keithley 616 digital electrometer (input impedance > 0.1 PΩ) that had been calibrated to within ±0.1 mV by using a Data Precision (model 8100) voltage calibrator. Fresh quinhydrone reference electrodes were prepared for each new experiment, which typically took less than 3 hr to perform. The standard midpoint potentials were obtained in two ways, either by placing various quinone/hydroquinone mixtures in the test half-cell or by titrating quinone solutions against ascorbic acid (Sigma). For these measurements, both aqueous HCl/KCl and 10% methanol/90% HCl/KCl were used, and these were deoxygenated by perfusion with pure nitrogen. The concentrations of the quinones, hydroquinones, and ascorbic acid in the test half-cell were determined spectroscopically by using a Beckman model 35 spectrophotometer in parallel experiments. The spectroscopic data obtained are given in Table 1. Depending on solubility, quinone concentrations of 0.1–0.6 mM were used.

The free radicals produced in the interaction of the various quinones with ascorbic acid were investigated by using a Varian E109 (ESR) spectrometer. For each measurement, a solution of 10 mM sodium ascorbate (Sigma) at pH 7.8 was flow mixed, using a vortex-flow mixing chamber (Varian E-249), with an aqueous 0.5 mM quinone solution. As with the electrochemical studies, solutions were deoxygenated by perfusion with pure nitrogen before mixing. The dynamics of the production and decay of the ascorbate and semiquinone radicals at 25°C was investigated for times between 10^{-1} to 10^{3} sec by using the stopped-flow technique. During these dynamic measurements, the spectrometer was locked to a characteristic absorption line of the ESR spectrum by using the Varian (model E-272B) field/frequency lock. Spin concentrations and *g* values were calibrated against aqueous $MnCl_2$ and Wurster's blue perchlorate ($g = 2.003$) solutions, respectively.

In the animal studies, female CD_1 mice (Charles River Breeding Laboratories) were inoculated with approximately 10^7 Ehrlich ascites tumor cells and randomized into groups of 10 mice each. This cell line is maintained in our laboratory by transplantation into CD_1 mice at 7-day intervals. Then, 24 hr after inoculation of the mice, the first of a 7-day course of twice-daily 0.25-ml intraperitoneal injections of a quinone/ascorbate mixture, which had been mixed only minutes earlier, was administered. On the 8th day, the mice were sacrificed and examined for the presence of ascites. The quinone doses were based on the following criteria: methoxy-, relatively nontoxic and soluble, the dosages used were well below lethal; 2,3- and 2,6-dimethoxy, dosages used were below those causing weight loss; 2,5-dimethoxy-, a saturated solution was used; benzoquinone was given in half the lethal dosage.

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* Permanent address: School of Electronic Engineering Science, University College of North Wales, Bangor, Gwynedd, United Kingdom.

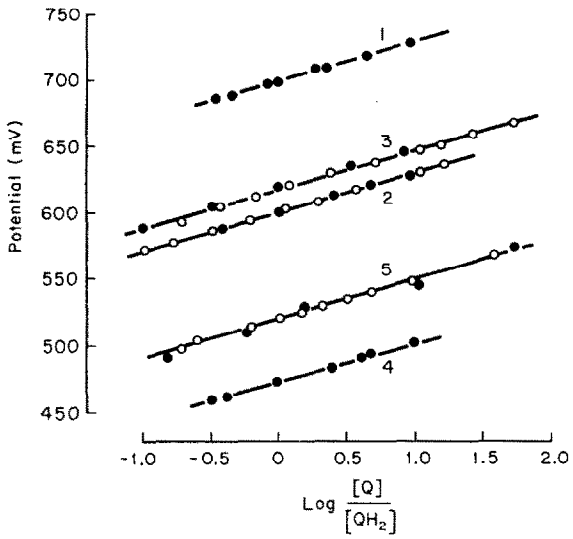

FIG. 1. Electrochemical potential at 25°C in 10% methanol/90% HCl/KCl as a function of the quinone (Q)/hydroquinone (QH_2) concentration ratio. ●, Quinone/hydroquinone mixtures; ○, quinone/ascorbic acid titrations. Curves: 1, *p*-benzoquinone; 2, methoxyquinone; 3, 2,3-dimethoxyquinone; 4, 2,5-dimethoxyquinone; 5, 2,6-dimethoxyquinone.

RESULTS

After conversion of the potentials measured with reference to the quinhydrone electrode to normal electrode potentials (hydrogen electrode, pH 0), the electrochemical data for the 10% methanol solutions were plotted in the form of the Nernst equation for the five quinones studied (Fig. 1). The slopes of the straight-line plots were found to be 29.5 ± 0.5 mV, consistent with the formation of two-electron redox couples. The standard midpoint potentials derived from these plots are given in Table 1, together with the results obtained using aqueous HCl/KCl. For the monomethoxy-, 2,3-, and 2,6-dimethoxy compounds, the quinone/hydroquinone mixtures gave results in good agreement with those of the ascorbic acid titrations. However, because ascorbic acid only partially reduced the 2,6-dimethoxy compound, the use of the spectroscopic data (Table 1) was required to determine the precise quinone/hydroquinone ratios. These results show that at pH 2 the dominant products formed from two-electron reduction of the quinones by ascorbic acid are the corresponding hydroquinones and dehydroascorbic acid. For 2,5-dimethoxy quinone (the least soluble compound studied), the titrations against ascorbic acid did not give consistent and reproducible electrochemical results. If, to maximize the balance, the highest concentration (≈0.5 mM) of the 2,5-dimethoxy compound was used in the titration, the electrochemical data did not produce a linear Nernst plot. Spectroscopic measurements for the range 0.05–0.5 mM gave inconsistent results, suggesting that factors such as dimerization or molecular association may have complicated the measurements. For this reason, the standard midpoint potential for the 2,5-dimethoxy compound was obtained solely from measurement of quinone/hydroquinone mixtures. It was also found that the reducing power of ascorbic acid toward the 2,5-dimethoxy was less than that for the 2,6-isomer. With increasing pH, the electrochemical measurements were more difficult to carry out. Problems associated with rapid oxidation of the hydroquinones increased with increasing pH and, for this reason, the electrochemical potentials given for pH 7.4 in Table 1 are based on adjustment of the pH 2 results.

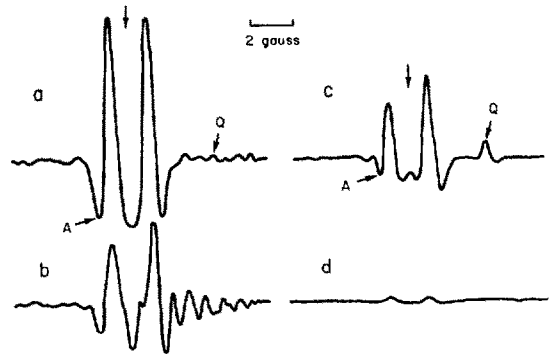

FIG. 2. ESR signals of quinone/ascorbate mixtures. Curves: a and b, ascorbate/2,6-dimethoxyquinone 5 and 25 sec after mixing; c and d, ascorbate/2,3-dimethoxyquinone 5 and 25 sec after mixing. A, spectral feature of the ascorbate radical used in kinetic studies; Q, spectral feature of the semiquinone radical used in kinetic studies; ↓, g = 2.0052.

We have also obtained spectroscopic data using chloroform as the solvent and our results for monomethoxy quinone and 2,6-dimethoxyquinone are in good agreement with those of Cosgrove *et al.* (5). We consider that the peak at 332 nm (ε = 1,670) reported for 2,6-dimethoxyquinone by Braude (11) and the extinction coefficient of 3,390 reported by Bu'Lock (12) for the peak around 370 nm for 2,5-dimethoxyquinone are in error.

Examples of the ESR signals obtained 5 and 25 sec after mixing of the quinones (pH 6) with sodium ascorbate (pH 7.8) are shown in Fig. 2 for 2,6- and 2,5-dimethoxyquinones. The pH of the mixtures was 7.6. The first signal to appear was that for the ascorbate free radical, and it was followed by that for the semiquinone radical; the identification and time courses of decay of these two radicals are shown in Figs. 2 and 3 and given in Table 2. We consider that the most significant result relevant

Table 1. Standard midpoint potentials (E_m) and spectroscopic data at 25°C

	E_m (pH 0)			λ_{max} (ε)*	
	HCl/KCl	90% MeOH/10% HCl/KCl	E_m (pH 7.4)	Quinone	Hydroquinone
p-Benzoquinone	699	700	261	296 (350), 248 (22,500)	290 (2,730)
Methoxyquinone	601	603	163	370 (1,400), 257 (16,300)	290 (3,390)
2,3-Dimethoxyquinone	621	622	183	400 (1,240), 257 (14,200)	284 (3,000)
2,5-Dimethoxyquinone	472	475	34	378 (540), 286 (21,500)	296 (3,400)
2,6-Dimethoxyquinone	516	521	78	390 (583), 285 (15,300)	290 (2,740)
Ascorbic acid†			47		

*In 10% MeOH/90% HCl/KCl.
†From ref. 10.

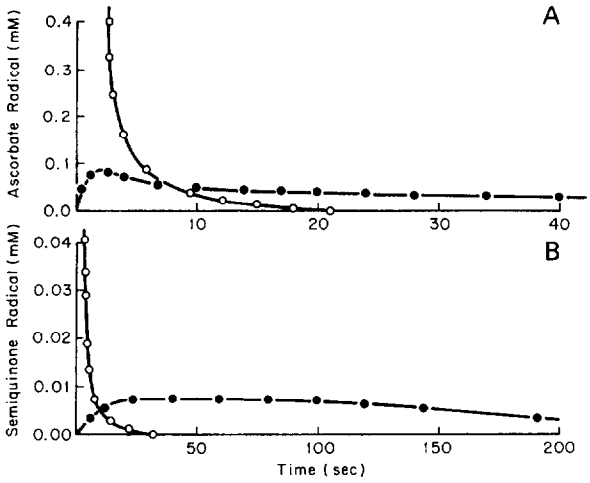

FIG. 3. Time course of decay of ascorbate (*A*) and semiquinone (*B*) free radicals produced by interaction of ascorbate with 2,3- (○) and 2,6- (●) dimethoxyquinones at 25°C and pH 7.6.

to the animal studies is that, in the reaction with sodium ascorbate, 2,6- and 2,5-dimethoxyquinones produced relatively stable semiquinone radicals and the longest lived ascorbate radicals. If semiquinone radicals were produced in the interactions with *p*-benzoquinone or with the methoxyquinone, then their lifetimes were less than 0.1 sec.

The results obtained to date in the determination of cytotoxic efficacy of the various quinone/ascorbate mixtures toward ascites cells are summarized in Table 3. Benzoquinone also was investigated, with and without ascorbate, and found to be inactive. Neither the quinones nor ascorbate when administered separately exhibited any appreciable cytotoxic activity, and only the 2,5- and 2,6-dimethoxyquinone/ascorbate mixtures were effective in eliminating Ehrlich ascites in a high percentage of the mice tested. Even from these incomplete studies with mice, there is a striking correlation between the cytotoxicity of the quinone/ascorbate mixtures and their ability to produce long-lived semiquinone radicals. Since at pH 7.4 the hydroquinones are likely to be rapidly oxidized to the corresponding quinones, hydroquinone/ascorbate mixtures were also given to the mice. The possible advantage of this was considered to be that production of the semiquinone radical would begin after, rather than before, injection of the mixture into the mice.

CONCLUSIONS

The electrochemical potentials given in Table 1 indicate that the two-electron reduction of 2,5- and 2,6-dimethoxyquinones by ascorbate will occur less rapidly than of the other quinones studied. This should favor the production of relatively stable radicals formed by one-electron reduction, in agreement with the ESR results. Because semiquinone radicals are more stable in basic than in acidic solution (13), the presence of transient one-electron reduction processes would not be expected to greatly modify the 29.5-mV gradient of the equilibrium Nernst plots of Fig. 1.

There appears to be a direct correlation between the cytotoxic action of the quinone/ascorbate preparations and the generation of long-lived semiquinone free radicals (Tables 2 and 3). Sodium ascorbate was used as the nontoxic electron donor in these studies. The results of this work predict that, if the molecular nature and hence the electrochemical potential of the electron donor is changed, then a corresponding modification of the quinone acceptor will be necessary to maintain the degree of one-electron reduction. This principle may have general application in the design of quinone-based anticancer drugs. The results presented here also lend support to the hypothesis (6) that semiquinone radicals have important cytotoxic properties. However, the real objective of our work is an increased understanding of living processes at the submolecular (electronic) dimension.

Table 2. Peak concentrations and half-lives of ascorbate and semiquinone radicals

	Peak concentration, mM		Half-life, sec	
	Ascorbate	Semiquinone	Ascorbate	Semiquinone
p-Benzoquinone	>0.5	NO	0.6	NO
Methoxyquinone	0.25	NO	4.5	NO
2,3-Dimethoxyquinone	0.81	0.069	1.7	3.0
2,5-Dimethoxyquinone	0.054	0.002	19.0	140.0
2,6-Dimethoxyquinone	0.081	0.008	21.0	195.0

NO, not observable after 0.1 sec.

Table 3. Summary of current results: Cytotoxic activity toward Ehrlich ascites-bearing mice of quinones with and without ascorbate

	Quinone, mM	Hydroquinone, mM	Ascorbate, mM	Mice tested, no.	% ascites free
Methoxy	3.6		750	10	10
	3.6			10	20
		18.0	750	20	0
		18.0		10	20
2,3-Dimethoxy	0.6		750	10	0
		0.6	750	10	0
		0.6		10	0
2,5-Dimethoxy	≈0.5*		750	10	50
		≈0.5*	750	10	60
		≈0.5*		10	0
2,6-Dimethoxy	3.0		750	10	100
	3.0			10	0
		1.5	750	60	83
		1.5		10	0
		1.5	375	20	95
		1.5	200	10	50
		1.5	100	10	10
Ascorbate alone			750	10	0
Control†				100	9

* Saturated.
† Saline injected.

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