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Free Radical Investigations as a Tool for the Study of Quinone Detoxification by Whole Cells and Enzymes

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Abstract

Populations of semiquinone and ascorbyl-free radicals with half-lives in excess of 10^3 s result from the interaction of 2,6-dimethoxy-*p*-quinone with ascorbate at pH 7.4. Viable cells and enzymes with reductive activity towards quinones and semiquinones are able to increase the rate with which these free radical populations decay. It is shown that the observation of the free radical decay kinetics can be used as an assay method on both whole cells and purified enzymes. The technique offers good sensitivity and the advantage that optical transparency is not required.

Introduction

Semiquinone-free radicals arising in the physiological milieu through enzymic activity or redox cycling [1] can lead to cellular damage [2] and exhibit mutagenicity [3]. A measure of protection against such toxicity is afforded to living systems by such enzymes as DT-diaphorase (EC 1.6.99.2) [1] which reduces quinones to less toxic hydroquinones, and superoxide dismutase (EC 1.15.1.1) [4] which detoxifies potentially damaging oxygen free radicals. There is interest not only in those agents capable of protecting living cells against the action of dangerous free radicals [5], but also in methods by which such toxic species can be purposefully generated under controlled physiological circumstances for chemotherapeutic applications [6, 7].

Previous experiments in this laboratory have demonstrated that solutions of some quinones administered in combination with ascorbate exhibit a potent cytotoxic action against Ehrlich ascites tumors in mice [8]. This synergistic cytotoxicity was found to be correlated with the production of long-lived populations of semiquinone and ascorbyl free radicals by one electron redox processes in the ascorbate-quinone mixtures. The most toxic of the combinations tested was 2,6-dimethoxy-*p*-quinone (2,6-DMQ) + ascorbate which produced a peak free radical concentration of about 4 μmol/l with a half life in excess of 10^3 s. The production of these radicals was independent of oxygen and transition metals [9], and no evidence for the presence of shorter-lived intermediates, such as hydroxyl radicals, was found by spin-trapping [9]. Direct studies on suspensions of a variety of cell lines employing electron spin resonance (ESR) spectroscopy have shown that viable cells have an ability to elimihate the free radical populations in ascorbate-quinone mixtures that varies significantly from one cell line to another [10, 11]. The elimination process involved an NAD(P)H-dependent enzyme(s) containing essential SH-groups [12].

In this article, the mechanism of free radical production in ascorbate-quinone mixtures and the means by which these radicals are eliminated by cells and enzymes are discussed. It is shown that the measurement of the decay kinetics of free radical populations offers a useful tool for the investigation not only of viable cells but also of enzymes. The significant difference observed by this free radical technique between normal and cancerous cell lines is discussed.

Experimental procedures

The technique employed for the production of free radicals was the same as reported earlier [8–12]. A solution of 20 mM sodium ascorbate (Sigma) containing 1 mg/ml superoxide dismutase (SOD, Sigma) and 5 mM ethylene glycol-*bis*-(α-amino-ethyl ether)*N,N,N',N'*-tetra-acetic acid (EGTA, Sigma) and one of 2 mM 2,6-dimethoxy-*p*-quinone (2,6-DMQ, kindly synthesized by Dr Gabor Fodor, University of West Virginia) were prepared in 50 mM HEPES buffer (pH 7.4).

At the start of the experiment, 0.25 ml of each solution was added to an additional 0.5 ml of buffer, and the mixture was transferred by syringe into the aqueous flat cell of the ESR spectrometer, as illustrated in Fig. 1. This instrument was equipped with a field-frequency lock that had been previously tuned to a resonance line of the radical to be investigated. The decay of the ESR line intensity was plotted as a function of time for 1400 s after the mixing of the ascorbate and quinone solutions. The individual kinetics of ascorbyl and semiquinone-free radical decays were determined in different

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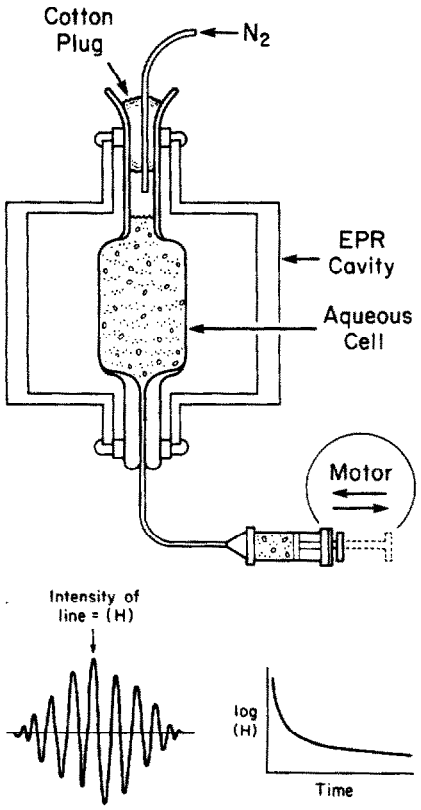

Fig. 1. Method of investigation of cell suspensions in an ESR spectrometer. A reciprocating syringe pump is used to keep the cell suspensions from settling.

experiments by adjusting the field-frequency lock to appropriate spectral resonance lines. For measuring the free radical decay kinetics of enzyme mixtures and cell suspensions, the ESR modulation was increased to 4 G so that a strong signal representing a weighted combination of the ascorbyl and semiquinone signals was obtained. This permitted the use of a shorter filter time constant and allowed extremely good reproduceability to be obtained in determinations of the decay kinetics. Such reproducibility allowed accurate measurements to be made even in situations where only a very small enhancement of the free radical decay rate over enzyme- or cell-free conditions occurred.

In experiments with viable cells, 0.25 ml of the ascorbate and 2,6-DMQ solutions (adjusted to a tonicity equivalent to 150 mM NaCl) were added to 0.5 ml of tissue culture medium containing the cells to be investigated. Cell concentrations of between 10^6 and 5×10^7 cells/ml were employed, the maximum concentration of cells of a given line being limited by their ability to eliminate the free radical populations. A syringe pump (see Fig. 1) was used to keep cell suspensions gently in motion while in the ESR instrument to prevent cells from settling out. The Ehrlich ascites cells were maintained in mice and harvested as described earlier [10]. Enzyme studies were done by placing double the required concentration of enzyme and substrates into 0.5 ml of HEPES buffer, and then adding 0.25 ml of each of the ascorbate and quinone solutions as before. The aqueous ESR cell was flushed through with oxygen-free nitrogen before the start of all experiments, and this atmosphere was maintained above reaction mixtures under investigation.

Results

The variation of the concentration of semiquinone-free radicals as a function of time after mixing ascorbate and 2,6-DMQ is shown in Fig. 2 and the corresponding kinetic for the ascorbyl-free radical concentration under the same reaction conditions is shown in Fig. 3. Examples of the decay kinetics corresponding to a weighted combination of the ascorbyl +semiquinone radical spectra achieved by broadening the modulation amplitude to increase the signal amplitude are illustrated at the top of Fig. 4. Plots a–f show the effect on the decay kinetic of increasing the number of Ehrlich ascites tumor cells in suspension in the ascorbate-quinone reaction mixture. A plot of the first-order rate constant for the linear portions of these decay loci as a function of the concentration of cells in suspension is shown at the bottom of Fig. 4.

The use of the free radical kinetic technique for enzymic measurements is illustrated in Fig. 5, where the first order rate constant for free radical decay is shown plotted as a function of the concentration of DT-diaphorase (assuming a molecular weight of 58000 Da) in the reaction mixture in the presence of 0.5 mM NADH. Neither DT-diaphorase alone nor NADH alone showed any reductive activity.

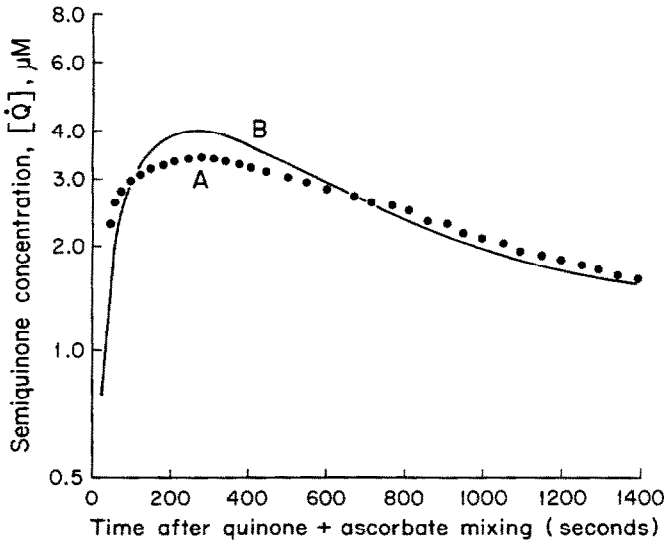

Fig. 2. (A) Experimentally determined kinetics of semiquinone-free radicals resulting from a solution of 0.5 mM 2,6-DMQ and 5 mM ascorbate at pH 7.4. The reagents were mixed at $t = 0$. (B) Theoretical fit to A (See text).

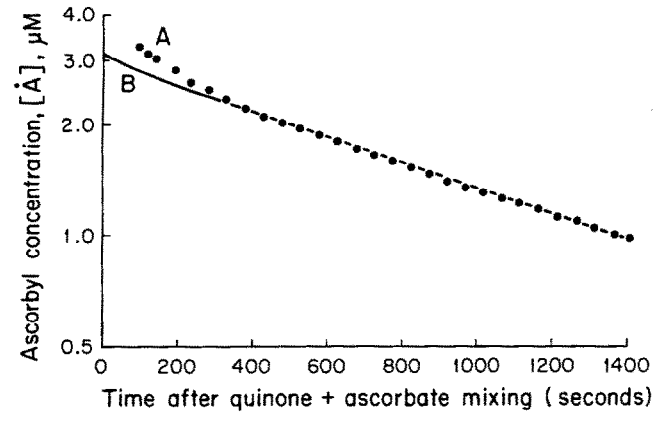

Fig. 3. (A) Experimentally determined kinetics for ascorbyl radicals under the same conditions as in Fig. 2. (B) Theoretical fit to A (See text).

Discussion

The overall reaction that occurs when ascorbate (A) is mixed with a quinone (Q) is the reduction of Q to form the corresponding hydroquinone (QH_2) with the concomitant oxidation of A to dehydroascorbate (DHA):

$$A + Q \rightarrow DHA + QH_2 \quad (1)$$

Although DHA is unstable under physiological conditions, this instability does not play a significant role in the early stages of the reaction kinetics to be discussed here and it will be ignored for the sake of simplicity. At thermodynamic equilibrium in reaction (1), the concentrations of the components will be related to the kinetic parameters k_1 and k_2 such that

$$k_1[A][Q] = k_2[DHA][QH_2] \quad (2)$$

The rate at which the reaction (1) will proceed will depend upon its displacement from equilibrium, so that

$$\frac{d[QH_2(t)]}{dt} = k_1[A][Q] - k_2[DHA][QH_2], \quad (3)$$

where $[QH_2(t)]$ is the hydroquinone concentration at time t after the start of the reaction. It can be shown [9] that the solution to this equation is

$$r(t) = 2c\{b + (b^2 - 4ac)^{\frac{1}{2}} \coth(kt)\}^{-1} \quad (4)$$

where

$r(t) = [QH_2(t)]/[Q_0]$,
$k = 0.5[Q_0]^2 k_1 (b^2 - 4ac)^{\frac{1}{2}}$,
$a = 1 - k_2/k_1$,
$b = 1 + [A_0]/[Q_0]$,
$c = b - 1$,

and $[A_0]$ and $[Q_0]$ are the initial ($t = 0$) concentrations of ascorbate and quinone in the reaction mixture, respectively.

Several processes can lead to the formation or interchange of free radicals:

$$Q + QH_2 = 2\dot{q} \quad (5)$$

$$A + DHA = 2\dot{a} \quad (6)$$

$$Q + A = \dot{q} + \dot{a} \quad (7)$$

$$Q + \dot{a} = \dot{q} + A \quad (8)$$

$$Q + 2A = 2\dot{a} + QH_2 \quad (9)$$

$$2Q + A = 2\dot{q} + DHA \quad (10)$$

It has been shown previously that the semiquinone-free radicals, $\dot{q}$, observed in the reaction mixture are generated by reaction (5), so that the concentration of semiquinone-free radicals present at any time will be given [13] by

$$[\dot{q}(t)] = (k_3[Q(t)][QH_2(t)])^{\frac{1}{2}} \quad (11)$$

where k_3 is pH dependent but otherwise constant. It follows from equation (4) that the time dependence of the semiquinone concentration should follow the kinetic

$$[\dot{q}(t)] = (k_3[Q_0]^2 r(t)[1 - r(t)])^{\frac{1}{2}} \quad (12)$$

Figure 2 shows the experimentally determined kinetics of the semiquinone concentration in a mixture of 2,6-DMQ and ascorbate (0.5 and 5 mM, respectively). The predicted kinetic is plotted for comparison, with $k = 1.667 \times 10^{-4}\ s^{-1}$ and $k_2/k_1 = 1.309$. Reasonable agreement is apparent, with the theoretical kinetic showing the characteristic build-up of semiquinone-free radicals and their subsequent slow decay. The discrepancy between theory and experiment in the early part of the kinetic may be due to ignoring the exchange reaction [equation (8)] when the difference in the concentrations of ascorbyl and semiquinone free radicals is very large (cf. Fig. 3).

The production of ascorbyl radicals, $\dot{a}$, cannot be accounted for by a similar conproportionation mechanism [9], and instead ascorbyl radicals arise as a direct consequence of 1-electron reductions of the quinone by ascorbate [reactions (7) and (9)], and decay by dismutation [reaction (6)]. Under these circumstances, the concentration of ascorbyl free radicals is expected [9] to follow the kinetic equation

$$[\dot{a}(t)] = k_4 r(t) - k_5 \int_0^t [\dot{a}(t)]^2 \, dt \quad (13)$$

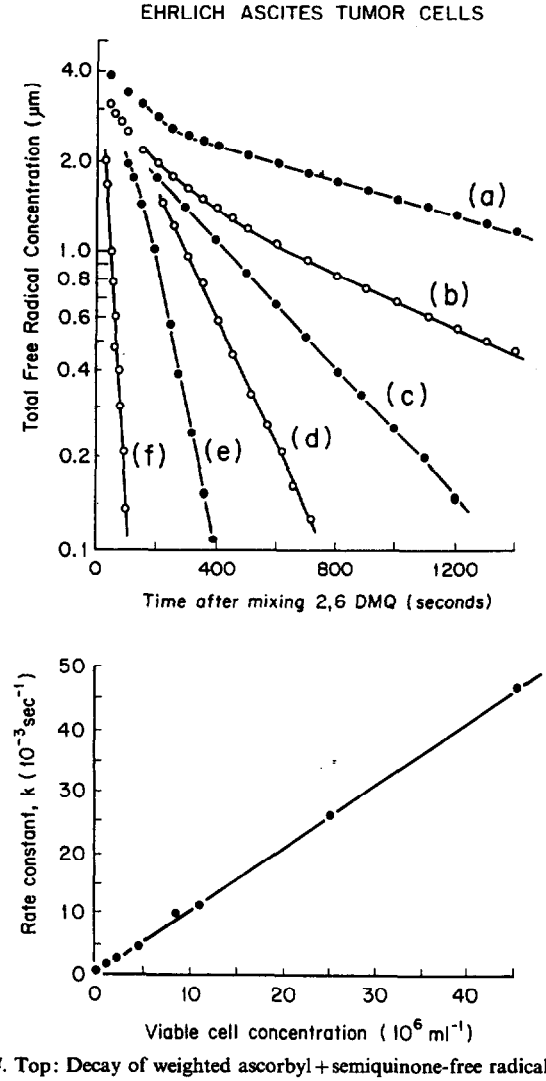

Fig. 4. Top: Decay of weighted ascorbyl + semiquinone-free radicals in the presence of Ehrlich ascites tumor cells. Viable cell counts were ($\times 10^6$ per ml): (a) 0.09, (b) 2.25, (c) 4.5, (d) 11.3, (e) 26, (f) 45. Bottom: Slope of linear portions of the above plots as a function of cell concentration.

Figure 3 compares the results obtained experimentally with the prediction of equation (13) with $k_4 = 0.8$ mM and $k_5 = 2 \times 10^{10}$ mol^{-1} s^{-1}. At times longer than about 200 s, reasonable agreement is again obtained.

It is of interest to consider the dependence of the reaction kinetics on the ascorbate and quinone concentrations in the reaction mixture. Since living cells and enzymes require reducing equivalents in the form of NAD(P)H in order to hasten the decay of the free radical populations (Figs. 4 and 5), it may be assumed that one (or more) of the products of reactions [(1) and (5)–(10)] is being chemically reduced in this process. Computer simulations using equations [(4), (12) and (13)] show that when the reaction is initiated with a 10-fold excess of ascorbate over quinone (as used experimentally), then conversion of any ascorbate oxidation products back to ascorbate results in only a very small enhancement in the overall reaction kinetics. On the other hand, the simulations show that the kinetics are very sensitive to reduction of either the quinone or semiquinone to form hydroquinone. The ability of cells and enzymes to cause a very rapid increase in the free radical decay kinetics must be related therefore to either the 1-electron reduction of semiquinone free radicals or else to the 2-electron reduction of the quinone, in each case to form the hydroquinone. The simulations show further that it is not possible to distinguish between these two processes by examination of the kinetics alone.

From these considerations it can be seen that the changes induced by living cells in the decay kinetics of free radical populations in ascorbate-quinone mixtures is a trace of the extent to which the cells are able to effect chemical reduction of the quinone and/or semiquinone. Any reductive action by the cells towards ascorbate reaction products will have a negligible effect on the measured kinetics.

The ability of cells to alter the free radical kinetics has been shown to result from enzymic activity [12]. Enzymes expected to be able to reduce quinones and/or semiquinones include oxido-reductase flavo-enzymes such as DT-diaphorase, lipoamide dehydrogenase, and glutathione reductase (mediated by glutathione) and thioredoxin reductase (mediated by thioredoxin). Figure 5 shows a typical result of an enzyme assay using the free radical kinetic technique. This result shows that the technique offers a detection limit of 0.1 nM for DT-diaphorase, or about 6 ng/ml, which offers adequate sensitivity for most assay purposes. The other flavoenzymes can also be detected at levels compatible with enzymic studies.

It should be noted that the ESR kinetic technique offers the advantage that cell suspensions can be investigated directly without regard to their optical properties. Typically, 5×10^6 cells are sufficient to test for activity, although several samples with different cell counts are desirable if a plot of rate constant versus cell count is to be constructed. Such cell numbers can be readily obtained both directly from living organisms and by tissue culture methods.

Earlier results demonstrated [11] that the free radical kinetics observed for several cell lines varied in parallel with their malignancy. In particular, a temperature-sensitive clone of normal rat kidney cells that exhibited a normal phenotype when cultured at 39 °C and a transformed phenotype when cultured at 33 °C showed a very much stronger activity against the ascorbate-quinone mixtures in the transformed state [11]. A similar result was obtained for Chinese hamster ovary cells, with those cultured in the absence of dibutyryl cyclic AMP (cells of a transformed phenotype) showing a much higher activity in the ESR assay than those cultured in its presence (cells of a normal phenotype) [11]. These results, which appear to reflect enzymic differences between normal and transformed cells, form the subject of current collaborative work between our laboratory and the M. D. Anderson Tumor Institute in Houston, Texas. If the cells of normal phenotype are incubated for 1 h in 50 μM dicumarol, evidence is found for the presence of DT-diaphorase since their ESR activity is reduced by this specific inhibitor. This response appears to be absent in the case of the transformed cells, however.

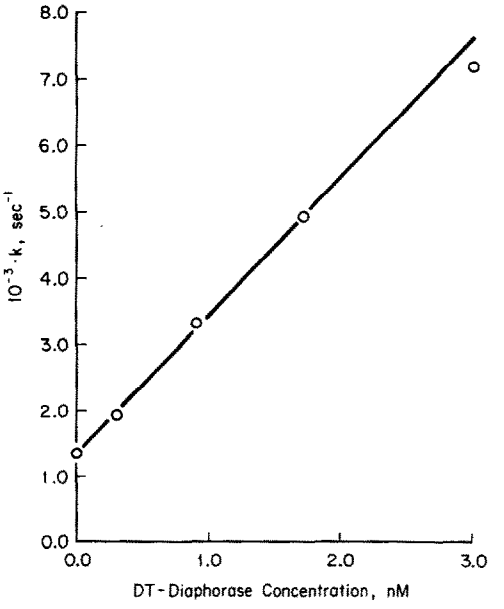

Fig. 5. First order rate constant for DT-diaphorase in the presence of 0.5 mM NADH.

Finally, although the computer simulations indicate that the free radical kinetics alone do not permit one to distinguish between 1- and 2-electron reductions in the ascorbate-quinone mixtures, there is an additional experimental technique that may allow for this distinction to be made. Measurements on Ehrlich ascites cells indicated that the kinetics of free radical decay were dependent on the ionic strength of isotonic solutions [10]. These results were interpreted as being due to coulombic repulsion between the negative electrical potential of the cell surface and the anionic free radical species which limited the rate with which they arrived at the cells, and hence the rate of the reaction. Since the majority of the 2,6-DMQ is neutral in charge at physiological pH, only the anionic free radicals would be affected by such a coulombic repulsion. It can be concluded that the Ehrlich ascites cells enhance the decay rate of the free radicals by direct action against semiquinone radicals. By contrast, another cell line (designated 54-5A4) that has a very high activity against the ascorbate-quinone solutions showed no such coulombic dependence as a function of ionic strength. It is probable, therefore, that this cell line was acting directly on the electrically neutral quinone by a 2-electron process.

Conclusions

The observation of the decay kinetics of free radicals offers a means for studying the enzymic reduction of quinones and semiquinones by suspensions of viable cells. The technique is

not dependent on the optical properties of the sample and the sensitivity makes it a viable alternative to other enzymic assay methods. Large differences are observed in the ability of normal versus cancerous cells to eliminate free radicals in this assay procedure. This difference may reflect significant variations in the oxido-reductase systems of normal and transformed cells.

Acknowledgements

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