Vol. 169, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1990 Pages 30-38

ON THE DETECTION OF PARAMAGNETIC SPECIES IN THE ADRIAMYCIN-PERFUSED RAT HEART: A REAPPRAISAL

B. Kalyanaraman[a]* and John E. Baker[b]

Departments of [a]Radiology and [b]Cardiothoracic Surgery,
Medical College of Wisconsin, 8701 Watertown Plank Road,
Milwaukee, Wisconsin 53226

Received April 10, 1990

SUMMARY. Recently, L. Costa *et al.* reported the direct detection of the superoxide anion and other paramagnetic species in the isolated, adriamycin-perfused rat heart [L. Costa *et al.* (1988) *Biochem. Biophys. Res. Commun.* 153, 275-280]. We have reevaluated the results of their study and concluded that the ESR parameters of the spectrum obtained from the adriamycin-perfused heart are consistent with that of the peroxyl radical and not with that of the superoxide anion. In addition, the ESR spectrum of the peroxyl radical is very likely produced as an artifact caused by the grinding of myocardial tissue. This artifact may mask the ESR spectra of the adriamycin-derived semiquinone radical and the iron-sulfur protein components of myocardium.

The cardiotoxicity of the antitumor drug adriamycin has been attributed to the formation of a semiquinone, a one-electron reduction metabolite, which undergoes redox-cycling in the presence of molecular oxygen to generate the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) [1-11]. In the presence of adventitious iron, the above sequence of reactions leads to the formation of a more powerful oxidant, either the hydroxyl radical or a perferryl species [12-14]. Adventitious iron may be present as a contaminant in the perfusate, or released from ferritin [15-17]. Both the superoxide anion and the adriamycin semiquinone have been shown to mobilize iron from ferritin in *in vitro* systems [15-17].

There is a considerable interest in the generation of oxy-radicals in the isolated, perfused heart in the context of ischemia and reperfusion [18-20]. Perfusion of the isolated rat heart with redox active quinones, such as adriamycin, has been shown to generate the hydroxyl radical, which was subsequently detected by ESR-spin trapping [21].

Costa *et al.* have recently reported the detection of the superoxide anion by direct ESR, in adriamycin-perfused rat hearts during low-flow ischemia [22]. The investigators employed a processing technique in which hearts, freeze-clamped to the temperature of liquid nitrogen, were ground under liquid nitrogen to a fine powder, transferred in ESR tubes to a liquid nitrogen Dewar, and the ESR spectra recorded at the temperature of liquid nitrogen [22]. It is now well established that mechanical grinding of frozen heart tissue can artifactually generate peroxyl and other radical species [23-26].

*To whom correspondence should be addressed.

Abbreviation used: **ESR**; Electron Spin Resonance.

0006-291X/90 $1.50

Due to the enormous interest in the free radical–mediated metabolism of drugs and xenobiotics in isolated organs [27–30], it is very likely that the methodology reported by Costa *et al.* may be adopted by other investigators. Thus, it is critical to determine whether their technique for tissue processing is valid,and to assess its impact upon the results reported in their study [22].

In this communication we have (i) compared the ESR parameters reported by Costa *et al* to the known data base available for oxy radicals, (ii) determined whether the radical species reported by Costa *et al* is generated as a consequence of adriamycin metabolism in the ischemic heart or is a peroxyl radical, generated artifactually during tissue processing of the adriamycin–perfused heart, and (iii) whether an artifactually generated peroxyl radical masks the appearance of authentic adriamycin semiquinone and the changes accompanying the iron sulfur protein associated with the mitochondrial NADH dehydrogenase, one of the enzymes proposed to be responsible for the metabolism of adriamycin in heart tissue [31–33].

MATERIALS AND METHODS

Isolated Heart Model. Adult Sprague Dawley rats, maintained on a standard diet, were used for this study. Anesthesia was induced and maintained with Halothane (4% and 1–2%, respectively), following which the right femoral vein was exposed and Heparin (150 IU/kg) administered intravenously. After 1 min., the chest was opened via median sternotomy and the pericardium removed. The aorta and inferior vena cavae were isolated, and the heart was rapidly excised and placed in ice–cold perfusion medium. After 30 sec., the aorta was attached to a stainless steel cannula, the pulmonary artery was incised to permit adequate coronary drainage, and the heart was perfused by the method of Langendorff [34] at a perfusion pressure equivalent to 12kPa (90 mm Hg). A three–way tap, located immediately above the site of cannulation, allowed the entire perfusate to be diverted away from the heart to produce global, no–flow ischemia. Rectal temperature in the rat was found to be 37° C. Normothermic perfusion was, therefore, carried out with perfusate at this temperature. The heart and perfusion fluids were kept in temperature–controlled chambers to maintain the myocardial temperature at the desired level. During all periods of ischemia, the heart was immersed in the perfusion medium used to infuse the coronary network.

Perfusion Medium. The standard perfusion fluid was Krebs–Henseleit bicarbonate buffer [35] having the following composition: 118.5 mM NaCl/25.0 mM $NaHCO_3$/4.8 mM KCl/1.2 mM $MgSO_4 \cdot 7H_2O$/1.2 mM KH_2PO_4 (pH 7.4 when gassed with 95% O_2/5% CO_2) in which the calcium content was reduced to 1.8 mM. To this was added 11.1 mM glucose. During the preparation of all calcium–containing solutions, precautions were taken to prevent the precipitation of calcium by gassing the solution with 5% CO_2. Before use, all perfusion fluids were filtered through cellulose acetate membranes of pore size, 5.0 μm.

Tissue Preparation. Immediately after mounting on the cannula, hearts (n = 8 per group) were perfused aerobically for a 10 min. equilibration period. Hearts (n = 8 per group) were then subjected to a 10 min. period of normothermic global ischemia. At the end of each perfusion period, hearts were freeze–clamped between stainless steel tongs (Biomedix, Elm Grove, WI) previously cooled to the temperature of liquid nitrogen. Frozen ventricular tissue was then processed for spectroscopic analysis by two separate methods. The first method of tissue processing involved grinding the frozen tissue to a fine powder using a stainless steel pestle and mortar (Biomedix) immersed in a liquid nitrogen bath. The fine powder was transferred under liquid nitrogen to a precision ESR tube (inside diameter, 3 mm; length, 30 mm) by placing the tube within the well of the mortar and dispensing the powdered tissue into the ESR tube with a spatula. The ESR tube was then immediately transferred to the lumen of a Dewar flask pre–cooled to the temperature of liquid nitrogen. The second method of tissue processing entailed chopping the wafer of frozen tissue under liquid nitrogen with a stainless steel spatula to produce small fragments ($\approx$ 2 mm cubes). These fragments were immediately transferred to the lumen of a Dewar flask pre–cooled to the temperature of liquid nitrogen. Sample sizes with the two methods were approximately equal. All flasks containing the processed heart tissue were then placed in the resonance chamber of the spectrometer.

Low–Temperature ESR of Adriamycin Semiquinone. Semiquinones from adriamycin were generated by anerobic metabolism of adriamycin catalyzed by xanthine and xanthine oxidase (Sigma Grade IV). A typical incubation

for ESR analysis consisted of adriamycin (100 μM), xanthine (400 μM), and xanthine oxidase (0.1 unit) in 2 mL phosphate buffer (100 mM, pH 7.5). Incubations were prepared in duplicate. Immediately after detecting the ESR spectra of the adriamycin semiquinone or adriamycin aglycone at room temperature, frozen icicles (3–4 cm) of the same incubation mixture were prepared and supported in the lumen of a finger Dewar filled with liquid nitrogen.

ESR Spectroscopy. ESR spectra from all samples were recorded at the temperature of liquid nitrogen using a Varian E–109 spectrometer at 9.5 GHz and 100–kHz field modulation. Magnetic field measurements were determined by using a Radiopan MJ–110 gaussmeter, and microwave frequency was measured by using an EiP 200 frequency counter. Hyperfine splitting was measured to 0.05 G directly from the magnetic field separations. All g value derivations were obtained from measurements of magnetic field and microwave frequency after correcting for the position of the gaussmeter probe.

RESULTS

Direct ESR parameters of the Superoxide Anion. In the previous report [Fig. 1b in ref.22] the ESR spectrum of freeze–clamped tissue from hearts perfused with adriamycin was assigned to the superoxide anion based on the g–values ($g_{\parallel} = 2.03$ and $g_{\perp} = 2.005$). The g–value of the superoxide anion is extremely sensitive to environmental factors such as complexation with metal ions, polarity of the medium, and temperature [36–38]. Table I lists the $g_{\parallel}$–values of the superoxide anion under a variety of experimental conditions. It is evident that the g–values ($g_{\parallel} = 2.03$ and $g_{\perp} = 2.005$) reported by Costa *et al.* do not correspond to the literature data base available for the

TABLE I
ESR PARAMETERS OF THE SUPEROXIDE ANION

Radical	Medium	$g_{\parallel}$ Values	Hyperfine Coupling (G)
$O_2^{\cdot-} - - Ba^{2+}$	H_2O/DMSO/$BaCl_2$[a]	2.095	–
		2.077	–
		2.065	–
$O_2^{\cdot-} - - Ca^{2+}$	H_2O/DMSO/$CaCl_2$[b]	2.095	–
		2.069	–
		2.048	–
$O_2^{\cdot-}$	H_2O	2.065	–
$O_2^{\cdot-}$	Ethanol	2.08	–
$O_2^{\cdot-}$	H_2O/$CaCl_2$	2.05	–
$^{\cdot}OOH$	H_2O	2.035	11.0

[a] At least three different superoxide species were detected by ESR, depending upon the concentration of Ba^{2+}. Although the structures of these species are not fully understood, it is believed that there is some type of complexation between $O_2^{\cdot-}$ and Ba^{2+}.

[b] Similar ESR spectra were obtained even in the presence of Ca^{2+}.

superoxide anion. Therefore, we conclude that the radical species responsible for Fig. 1b in ref. 22 could not be the superoxide anion.

Artifactual Generation of the Peroxyl Radical in Ground Myocardial Tissues. It is well known that radical species can be generated artifactually in frozen tissues by mechanical grinding [39,40]. We and others have verified that this effect is present in heart tissue [23–26]. The ESR spectra from the rat heart subjected to 10 min. of ischemia, freeze–clamped with the frozen tissue chopped or ground are completely different (Fig. 1). The multi–line ESR spectrum of ground tissue has previously been analyzed to consist of these components a semiquinone, possibly a ubisemiquinone radical ($g_{iso} = 2.004$), a peroxyl radical ($g_{\parallel} = 2.04$), and an unidentified radical ($g_{iso} = 2.002$) [23]. At higher temperatures ($\simeq$ 173 K), only the absorptions due to the peroxyl radical and the ubisemiquinone were evident (Fig. 1). From these results, we conclude that the ESR spectral components (Fig. 1 in ref. 22) previously assigned to the superoxide anion should be reassigned to the peroxyl radical species. In addition, the peroxyl radical spectrum has been generated as a consequence of mechanical manipulation of the adriamycin–perfused heart tissue, and not as a free radical intermediate arising from the metabolism of adriamycin.

ESR Parameters of Adriamycin Semiquinone at Low Temperatures. In their paper, Costa *et al.* [22] attributed the increase in oxy–radicals in adriamycin–perfused hearts during low–flow ischemia to the redox–cycling of

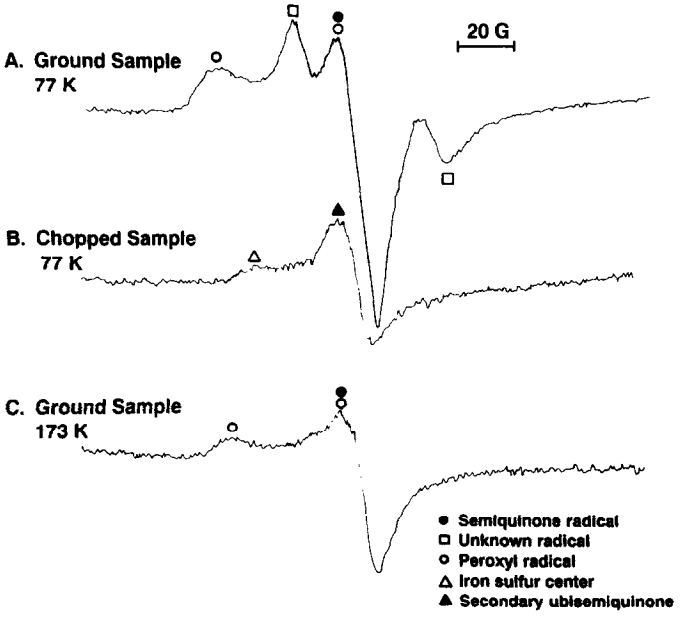
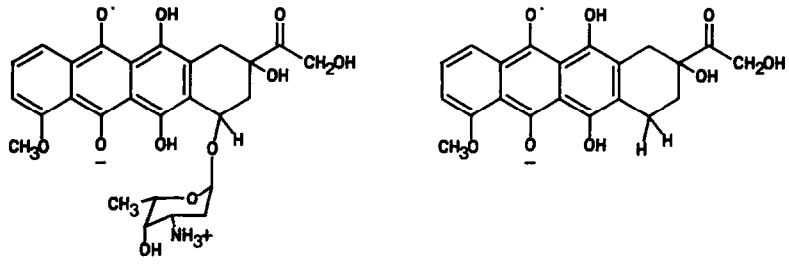


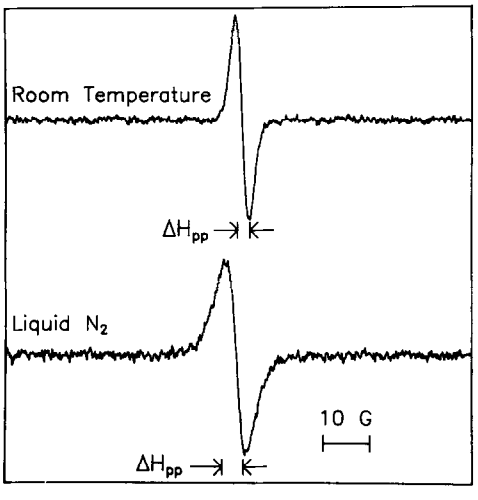
Figure 1. ESR spectrum of ischemic rat heart freeze–clamped with the frozen tissue ground (A) or chopped (B) prior to recording of spectrum. The temperature of the sample was measured to be 77 K. The ground sample was then warmed to 173 K (C) prior to recording of spectrum. Spectrometer conditions: microwave power, 2 mW; modulation amplitude, 2.0 G; scan range, 200 G.



I II

Figure 2. The molecular structures of adriamycin semiquinone (I) and adriamycin deoxyaglycone semiquinone (II).

semiquinones derived from ubiquinone and adriamycin. However, neither species were detected in ground myocardial tissue. To our knowledge, the existence of low-temperature (77 K) ESR spectra derived from semiquinone radicals formed from one-electron reduction of adriamycin has not been reported. In order to better



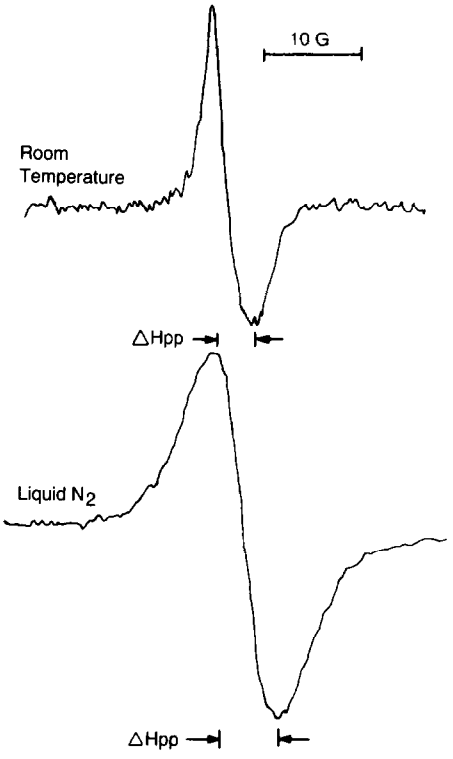
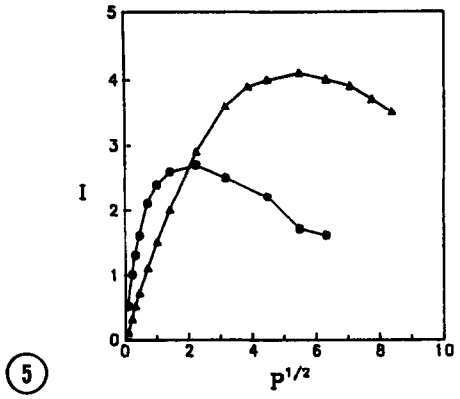
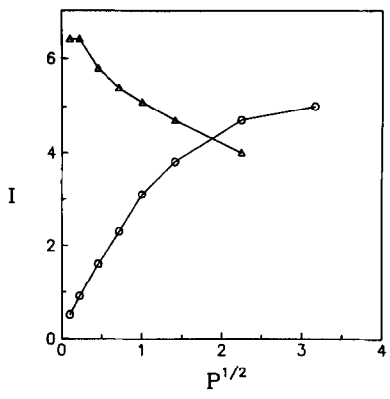


Figure 3. ESR spectra of adriamycin semiquinone radical I. This radical was generated according to experimental conditions described under Materials and Methods. Spectrometer conditions: microwave power 1 mW; modulation amplitude 1 G; scan range 100 G.

Figure 4. ESR spectra of adriamycin deoxyaglcone semiquinone II. The ESR spectrum of radical II appeared, with time, from the same incubation mixture in Fig. 3.



(5)



(6)

Figure 5. Relationship between the microwave power (P) in mW and the signal intensity (I) of adriamycin semiquinone at room temperature (▲) and at liquid nitrogen temperature (●).

Figure 6. Relationship between the microwave power (P) in mW and the signal intensity (I) of adriamycin deoxyaglycone at room temperature (○) and at liquid nitrogen temperature (△).

understand the ESR spectra of these species in freeze–clamped tissues, we have characterized their spectra using the xanthine / xanthine oxidase model system.

Adriamycin semiquinone (I) and adriamycin deoxyaglycone semiquinone(II) (Fig. 2) were generated by the reductive metabolism of adriamycin at room temperature in a nitrogen saturated environment using the xanthine / xanthine oxidase system. The characteristic symmetrical one–line spectrum of I (Fig. 3, room temperature) was first observed, which changed with time to the asymmetrical ESR spectrum corresponding to II (Fig. 4, room temperature). Rapid freezing of incubation mixtures at room temperature in Figs. 3 and 4 gave the liquid nitrogen ESR spectra of I and II as shown in Figs. 3 and 4.

The peak–to–peak line widths (ΔH_{pp}) of I are 3.25 G (room temperature) and 4.25 G (77 K), and ΔH_{pp} of II are 4.25 G (room temperature) and 6.5 G (77 K), respectively.

Power saturation curves of I and II at room temperature were different from those obtained at liquid nitrogen temperatures (Figs. 5 and 6). Comparison of the low temperature ESR parameters of the adriamycin semiquinone with that of the endogenous ubisemiquinone radical [23] reveal considerable differences with respect to these ESR parameters.

ESR Parameters of the Iron–Sulfur Protein in Myocardial Tissue. The ESR spectrum of chopped ischemic heart samples reveals the presence of a ubisemiquinone or a flavine semiquinone ($g = 2.0045$) and a reduced iron–sulfur component ($g = 2.02$) (Fig. 1B). When the scan range was increased to 1000 G, an additional peak ($g = 1.94$) due to the iron–sulfur center was observed. These iron–sulfur center peaks are associated with the mitochondrial NADH or succinate dehydrogenase [41,42].

DISCUSSION

This study demonstrates that the ESR parameters reported by Costa *et al* [22] correspond to that of the peroxyl radical, not to that of the superoxide anion as interpreted in their study. The peroxyl radical is generated

artifactually by mechanical grinding of frozen adriamycin–perfused heart tissue, not as an intermediate formed during the metabolism of adriamycin in the ischemic heart. Furthermore the artifactually generated peroxyl radical masks the appearance of the authentic adriamycin–semiquinone radical.

The adriamycin semiquinone radical is only detectable by ESR under totally anaerobic conditions. At oxygen concentrations in excess of 1 μM, the half–life for this radical species has been estimated to be approximately 6 ms [6]. The intracellar oxygen concentration in heart tissue perfused with adriamycin at low flow rates (i.e., 1 ml/min.) is far greater than 1 μM. Therefore, it is not feasible to detect this radical species in heart tissue using the freeze–clamping technique previously reported [22]. However, it may be possible to detect the adriamycin semiquinone in freeze–clamped heart tissue, perfused with adriamycin and then subjected to no–flow global ischemia (when the oxygen supply is completely cut off). The half–life of the adriamycin semiquinone radical is also increased considerably under anaerobic conditions because of the disproportionation–coproportionation equilibrium between the adriamycin and its hydroquinone [6].

It has been shown that NADH–dependent lipid peroxidation of bovine heart mitochondria is catalyzed by NADH dehydrogenase in the respiratory chain via transfer of electrons from the dehydrogenase to iron–chelates [43]. Adriamycin metabolism has been shown to be catalyzed by the reduced iron sulfur center 1 associated with NADH dehydrogenase in submitochondrial particles [31,32]. The intensity of the ESR absorption peaks at g = 2.025 and 1.94 may be considerably reduced in chopped ischemic heart tissue freeze–clamped after perfusion with adriamycin. It should, however, be cautioned that mechanical grinding will make it virtually impossible to detect changes in the region $g = 2.025$ because of the overlap of the ESR signal from the artifactually–generated peroxyl radical.

Radical species may be formed by the breakage of the unstable oxygen–oxygen bond of lipid hydroperoxides during mechanical grinding of frozen myocardial tissue [24]. *In vitro* microsomal studies have shown that the extent of lipid peroxidation induced by adriamycin is the greatest under hypoxic conditions [17,44,45]. Based on this, it is conceivable that the increase in the ESR spectral intensity in adriamycin–treated heart tissues, as detected by Costa *et al.* is probably due to greater production of the artifactual peroxyl radical in adriamycin–perfused hearts as compared to control hearts.

In conclusion, we have reiterated that the experimental technique used by Costa *et al.* [22] is prone to artifacually generate the peroxyl radical and have rectified their spectral misinterpretations. The use of frozen heart samples, processed by chopping, prior to spectroscopic analysis may be of use in the development of future strategies designed to investigate free radical–mediated adriamycin metabolism in myocardium.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health Grants HL–43030, GM–29035 and RR–01008.

REFERENCES

1. Handa, K. and Sato,S. (1975) *Gann* **66**, 43–47.

2. Bachur, R. R., Gordon, S. L. and Gee, M. V. (1977) *Mol. Pharmacol.* **13**, 901–910.

3. Bachur, N. R., Gordon, S. L., and Kon, H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 954–957.

4. Goodman, J., and Hochstein, P. (1977) *Biochem. Biophys. Res. Commun.* **77**, 797–803.

5. Gutierrez, P. L., Gee, M. V., and Bachur, N. R. (1983) *Arch. Biochem. Biophys.* **223**, 668–75.

6. Kalyanaraman, B., Perez–Reyes, E., and Mason, R. P. (1980) *Biochim. Biophys. Acta* **630**, 119–130.

7. Powis, G., and Appel, P. A. (1980) *Biochem. Pharmacol.* **29**, 2567–2572.

8. Thayer, W. S. (1977) *Chem. Biol. Interactions* **19**, 265–278.

9. Doroshow, J. H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4514–4518.

10. Lown, J. W. (1985) *Adv. Free Radical Biol. Med.* **1**, 225–264.

11. Doroshow, J. H. (1983) *Cancer Res.* **43**, 460–472.

12. Winterbourn, C. C. (1987) *Free Radical Biol. Med.* **3**, 33–39.

13. Rush, J. D. and Koppenol, W. H. (1986) *J. Biol. Chem.* **261**, 6730–6733.

14. Winterbourn, C. C. and Sutton, H. C. (1984) *Arch. Biochem. Biophys.* **235**, 116–126.

15. Thomas, C. E. and Aust, S. D. (1986) *Arch. Biochem. Biophys.* **248**, 684–689.

16. Thomas, C. E., Moorehouse, L. A., and Aust, S. D. (1985) *J. Biol. Chem.* **260**, 327–3280.

17. Vile, G. F. and Winterbourn, C. C. (1988) *Biochem. Pharmacol.* **37**, 2893–2897.

18. Hearse, D. J. (1977) *J. Mol. Cell Cardiol.* **9**, 605–616.

19. McCord, J. M. (1985) *New Engl. J. Med.* **312**, 159–163.

20. Hess, M. S. and Manson, N. H. (1984) *J. Mol. Cell Cardiol.* **16**, 969–985.

21. Rajagopalan, S., Politi, P. M., Sinha, B. K., and Myers, C. E. (1988) *Cancer Res.* **48**, 4766–4769.

22. L. Costa, L., Malatesta, V., Morazzoni, F., Scotti, R., Monti, E., and Parracchini L. (1988) *Biochem. Biophys. Res. Commun.* **153**, 275–280.

23. Baker, J. E., Felix, C. C., Olinger, G. N., and Kalyanaraman, B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2786–2789.

24. Baker, J. E., Felix, C.C., and Kalyanaraman, B. (1988) in *Oxy–Radicals in Molecular Biology and Pathology*, (P. Cerutti, I. Fridovich, J. M. McCord, eds.) Alan R. Liss, Inc. pp.343–351.

25. Nakazawa, H. Ichimori, K., Shinozaki, Y., Okino, H., and Hori, S. (1988) *Am. J. Physiol.* **255**, H213–H214.

26. Garlick, P. G., Shuter, S. L., Davies, M. J., Hearse, D. J., and Slater, T. F. (1988) *J. Mol. Cell Cardiol.* **20**, S78.

27. Bador, M. Z., Graney, P. E., Yoshihara, H., Kauffman, F. C., and Thurmann, R. G. (1989) *J. Pharmacol. Exp. Ther.* **248**, 1317–1322.

28. Sies, H. and Cadenas, E. (1985) *Phil. Trans., R. Soc. Lond B* **311**, 617–631.

29. Sinha, B. K. (1987) *Biochim. Biophys. Acta* **924**, 261–269.

30. Connor, H. D., Thurman, R. G., Galizi, M. D., and Mason, R. P. (1986) *J. Biol. Chem.*, **261**, 4542–4548

31. Davies, K. J. A., and Doroshow, J. H. (1986) *J. Biol. Chem.* **261**, 3060–3067.

32. Davies, K. J. A., and Doroshow, J. H. (1986) *J. Biol. Chem.* **261**, 3068–3074.

33. Nohl, H. (1987) *FEBS Lett.* **214**, 269–273.

34. Langendorff, O. (1985) *Pflugers. Arch. Ges. Physiol.* **61**, 291–332.

35. Krebs, H.A. and Henseleit, K. (1932) *Hoppe–Seyler's Z. Physiol. Chem.* **210**, 33–66.

36. Bray, R. C., Mautner, G. N., Fielden, E. M., and Carle, C. I. (1977) in *Superoxide and Superoxide Dismutases* (eds. A. M. Michelson, J. M. McCord, and I. Fridovich), Academic Press, N. Y., pp. 61–75.

37. Knowles, P. F., Gibson, J. F., Pick, F. M., and Bray, R. C. (1969) *Biochem. J.* **111**, 53–58.

38. Symons, M. C. R. (1985) *Phil. Trans., R. Soc. Lond. B* **311**, 451–472.

39. Benedetto, C., Bocci, A., Dianzani, M.U., Ghiringhello, B., Slater, T.F., Tomasi, A., and Vannini, V. (1981) *Cancer Res.* **41**, 2936–2942.

40. Benedetto, C. (1982) In *Free Radicals, Lipid Peroxidation, and Cancer,* eds McBrien, D.C.H. and Slater, T.F. (Academic, New York), pp 27–54.

41. Orme–Johnson, N. R., Orme–Johnson, W. H., Hansen, R. E., Beinert, H., and Hatefi, Y. (1973) in *Oxidases and Related Redox Systems,* (T.E. King, H. S. Mason and M. Morrison, eds.) University Park Press, Vol.2, pp. 769–797.

42. Orme–Johnson, N. R., Hansen, R. E., and Beinert, H. (1974) *J. Biol. Chem.* **249**, 1928–1939.

43. Takeshige, K., Takayanagi, R., Minakami, S. (1980) *Biochem. J.* **192**, 861–866.

44. Mimnaugh, E. G., Trush, M. A., and Gramm, T. E. (1988) *Biochem. Pharmacol.* **30**, 2797–2804.

45. Mimnaugh, E. G., Trush, M. A., and Gramm, T. E. (1983) *Cancer Treat. Rep.* **67**, 731–733.