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Reactions of the Antitumor Agent Carminic Acid and Derivatives with DNA

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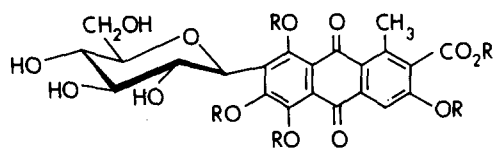
The antitumor agent carminic acid **1a** does not bind to DNA but nicks it slowly, more rapidly when reduced *in situ*, and still more rapidly when prereduced at the quinone moiety. The nicking process requires oxygen and is selectively inhibited by (i) superoxide dismutase, (ii) catalase, and (iii) free radical scavengers indicating the involvement of $O_2^{\cdot}$, H_2O_2, and $OH^{\cdot}$, respectively. The intermediacy of $OH^{\cdot}$ was supported by spin trapping with *N*-t-butyl-α-phenylnitrone and epr of the radical produced *via* the carminic acid semiquinone. The single strand scission of DNA by carminic acid requires two adjacent hydroquinone moieties in the chromophore since reduced methyl tetra-*O*-methylcarminate **1b** is without effect although it binds weakly to DNA. Polarographic redox potentials for the reversible ($2e$, $2H^+$) reduction of **1a** and **1b** are -0.736 ± 0.003 V and -0.56 ± 0.010 V against SCE, respectively. The fact that daunorubicin and adriamycin produce more extensive DNA strand scission than carminic acid under comparable conditions of prereduction and on a molar basis is largely attributed to the assistance of intercalative binding afforded in the case of the anthracyclines.

INTRODUCTION

Carminic acid **1a** (Fig. 1). the principal component of the food dye cochineal, obtained from the insect *Nopalea coccinellifera* (*1*) shows significant inhibitory activity against ascites tumors in Jensen rats (*2*). To date no studies have been reported on its mode of action. The inhibition of tumor growth by carminic acid and its structural similarity to the antitumor agent shikonin (*3*) and the anthracyclines, which react preferentially with nucleic acids (*4*), suggested a similar cell target site for carminic acid.

We report studies of the reactions of carminic acid, its reduced form, and methyl tetra-*O*-methyl carminate **1b** with DNA together with those electrochemical studies of these compounds which pertain to their redox characteristics.



1a (R=H)

1b (R=CH_3)

FIG. 1. Structural formulae for carminic acid **1a** and methyl tetra-*O*-methylcarminate **1b**.

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EXPERIMENTAL

Materials. Carminic acid **1a** was obtained from Fisher Scientific Co. and was purified via the barium salt followed by acidification and recrystallization from methanol pet. ether (40–60°C), mp, 136°C (*5*). Methyl tetra-*O*-methylcarminate **1b** was prepared by treatment of carminic acid with excess ethereal diazomethane in anhydrous methanol (*6*) yellow needles; mp, 185–188°C (from C_6H_6:petroleum ether). *N*-t-Butyl-α-phenylnitrone was from Aldrich Chemical. Superoxide dismutase (EC 1.15.1.1) was from Miles Labs, and catalase (EC 1.11.1.6) (beef liver) was from Aldrich Chemical Co. Ethidium bromide and disodium EDTA[2] were purchased from Sigma. PM2-CCC-DNA (88% CCC) was prepared as described before (*7*).

Melting points were determined on a Fisher Johns apparatus and are uncorrected. The ir spectra were recorded on a Nicolet 7199 F.T. spectrophotometer, and only the principal sharply defined peaks are reported. The nmr spectra were recorded on Varian A-60 and A-100 analytical spectrometers. The spectra were measured on approximately 10–15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as standard. Line positions are reported in parts per million from the reference. First-derivative epr spectra were measured on an instrument containing a Bruker EA-400 EPR console with a Varian V360-1 12-in. magnet and VFR 2503 Hall effect controller operating at a nominal frequency of 9.3 GHz. The microwave power incident on the cavity was attenuated to 10 db below maximum. Hyperfine couplings and *g*-values were measured by direct fieldial measurements.

Keisgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin-layer chromatography.

Ethidium fluorescence assay for nicking of DNA. The fluorimetric methods of measuring strand breakage of PM2 covalently closed circular DNA (CCC-DNA) and its inhibition by enzymes and free radical scavengers has been described (*7*). The conversion of PM2-CCC-DNA to nicked open circular DNA results in a 30% increase in fluorescence in the pH 11.8 ethidium assay solution (which was 20 μM potassium phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 μg/mol of ethidium bromide) and 100% loss of fluorescence after a 96°C heating and 23°C cooling cycle since the strands are now separable. The reactions were performed at 37°C in a volume of 200 μl containing 50 mM potassium phosphate, pH 7.0, 1.02 A_{260} units of PM2-CCC-DNA (85% CCC), 6×10^{-5} M carminic acid, 5.3×10^{-3} M of sodium borohydride, and 10% dimethyl sulfoxide and other components as indicated in the legends to the figures.

Polarographic determination of redox potentials. Polarographic analysis of carminic acid and its pentamethyl derivative were carried out as described previously (*8*) using a Princeton Applied Research Model (P.A.R.) 174 polarograph and P.A.R. accessories. The drop time was controlled at 2 sec. All potentials were measured and are reported with respect to the aqueous saturated calomel electrode.

RESULTS AND DISCUSSION

Carminic acid (6×10^{-5} M) subjected to *in situ* reduction of the chromophore with 5.3×10^{-3} M sodium borohydride [as indicated by a change in the absorption

[2] Abbreviations: CCC, covalently closed circular; OC, open circular; EDTA, ethylenediaminetetraacetic acid, disodium salt; A_{260}, absorbance at 260 nm.

maximum from 500 (ε, 6000) to 400 nm (ε, 14 666)] nicks 52% of PM2-CCC-DNA in 60 min (Fig. 2). Treatment of the DNA with prereduced carminic acid at the same concentration produces 92% nicking of the DNA in 60 min. Unlike the structurally related anthracyclines, carminic acid alone causes a small (~10%) but reproducible

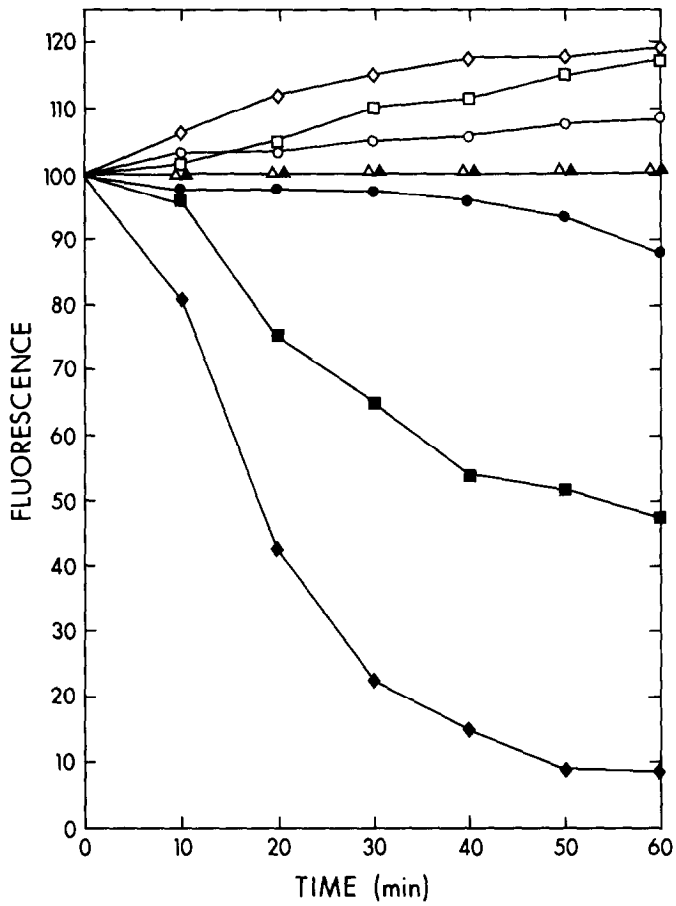

FIG. 2. Single-strand scission of PM2-CCC-DNA by reduced carminic acid. Reactions were performed at 37°C in 0.05 *M* potassium phosphate buffer, pH 7.0, and contained 1.0 A_{260} unit/ml of PM2-CCC-DNA (85% CCC). Aliquots (20 μl) at the indicated times were added to 2.3 μl of the standard pH 11.8 ethidium bromide assay mixture. The before-heat fluorescence readings are shown with open symbols, and the closed symbols are fluorescence readings after the denaturation at 96°C for 3 min followed by rapid cooling. Additional components were (△——△) 5.3×10^{-3} $NaBH_4$ or 6×10^{-5} *M* methyl tetra-*O*-methyl-carminate and 5.3×10^{-3} *M* $NaBH_4$; (○——○) 6×10^{-5} *M* carminic acid; (□——□) 6×10^{-5} *M* carminic acid and 5.3×10^{-3} *M* $NaBH_4$; (◇——◇) 6×10^{-5} *M* carminic acid prereduced with 5.3×10^{-3} *M* $NaBH_4$.

nicking of the DNA in 60 min. The nicking requires oxygen and is inhibited by (i) catalase, (ii) superoxide dismutase, and (iii) free radical scavengers such as isopropyl alcohol or sodium benzoate (Fig. 3). The selective effects of the enzymes strongly implicate the intermediacy of $O_2^{\cdot-}$ and H_2O_2, while the inhibition of cleavage by free-radical scavengers suggest the intermediacy of the $OH^{\cdot}$ radical. The results also indicate

that the DNA cleavage is due to the attack by OH· radicals (*9*) formed by reoxidation of the hydroquinone form of carminic acid via the semiquinone.

Further support for the intermediacy of the hydroxyl radical was obtained by spin trapping (*10*) experiments in conjunction with epr spectroscopy. Exposure of the reduced form of carminic acid to air generated the semiquinone species which was

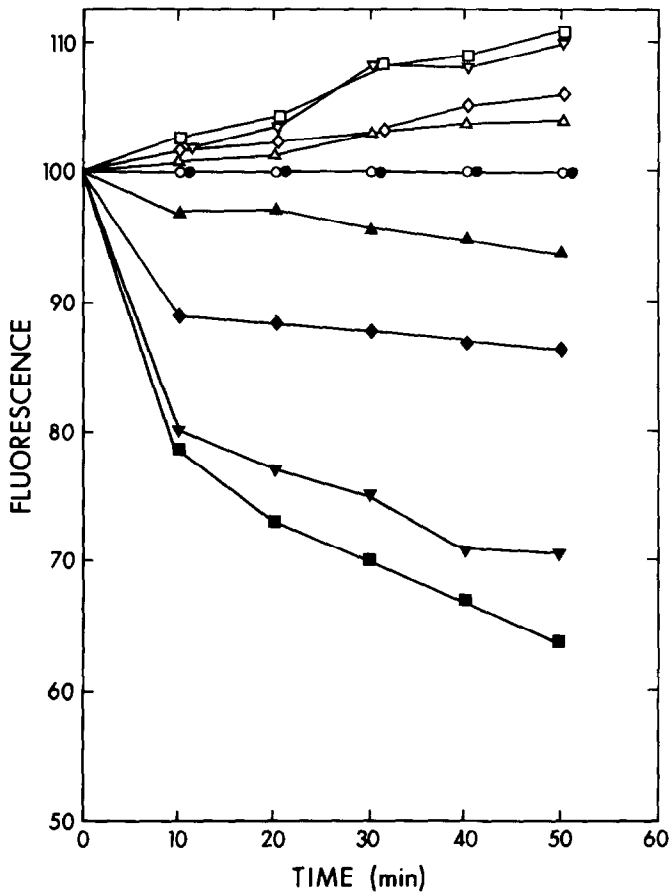

FIG. 3. Single-strand scission of PM2-CCC-DNA by quinone-reduced carminic acid and its selective enzymatic inhibition. Reactions were performed using the conditions described in the legend for Fig. 2 but with the following additional components: (□——□) none; (○——○) 0.6 *M* isopropyl alcohol; (△——△) 0.02 *M* sodium benzoate; (◇——◇) 5×10^{-6} g/ml of catalse; (▽——▽) 5×10^{-5} g/ml of superoxide dismutase.

determined by epr (Fig. 4a) to have appreciable lifetime under these conditions. Addition of the spin-trap *N*-t-butyl-α-phenylnitrone (PBN) in 50% aqueous dimethyl sulfoxide resulted in the generation of the epr spectrum of the OH·-trapped nitroxide radical (Fig. 4b) with the characteristic three doublet splitting of 15.9 ± 0.2 and 3.0 ± 0.2 G (*11*, *12*). Although some yield of the radical species is sacrificed, owing to the tendency of dimethyl sulfoxide to act as a trapping agent, this was more than offset by the increased lifetime of the PBN · OH nitroxide and the diminished dielectric loss in this

solvent relative to water. The hyperfine splittings observed under these solvent conditions agreed with that of the PBN–OH adduct obtained when the OH· radical was generated photochemically from hydrogen peroxide and trapped with the nitrone (*11*, *12*). By contrast, when an aliquot of the DMSO solution of carminic acid semiquinone was added to 0.2 *M* PBN in 50 m*M* potassium phosphate buffer, pH 7.2, containing 1 mg/ml of catalase, or superoxide dismutase and catalase, no PBN · OH radical could be detected confirming that H_2O_2 and $O_2^{\cdot-}$ are required in the generation of the OH· radical. Strictly speaking, observation of the PBN–OH adduct is not absolute proof of the intermediacy of OH· since it merely indicates that the PBN has been oxidized. Nevertheless the cumulative evidence for the OH· radical is compelling.

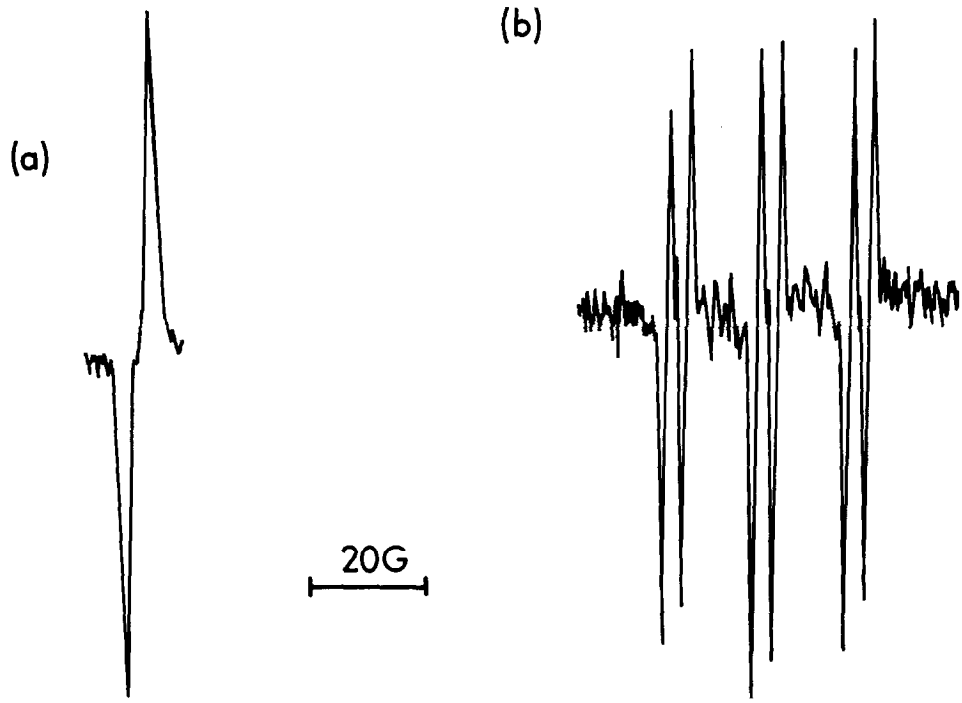

FIG. 4. (a) Electron paramagnetic resonance spectrum (microwave power, 22 db; modulation amplitude, 0.2 G) of carminic acid semiquinone obtained immediately after mixing 5×10^{-3} *M* carminic acid and 10^{-2} *M* sodium borohydride in moist dimethylsulfoxide. $g = 2.0037$. (b) Electron paramagnetic resonance spectrum (microwave power, 6 db; modulation amplitude, 0.32 G) of the PBN-OH radical adduct obtained after incubation of 5×10^{-3} *M* carminic acid, 10^{-2} *M* sodium borohydride, 0.1 *M* phenyl-t-butylinitrone (PBN), and 0.05 *M* potassium phosphate buffer, pH 7.2, in 50% aqueous dimethylsulfoxide for 4 hr. $a^N = 15.9$ G; $a_\beta^H = 3.0$ G, $g = 2.0059$.

These results point to the following overall mechanism [steps (1–7)] for generation of radicals and subsequent cleavage of DNA:

$$CA + [2H] \rightarrow CAH_2 \quad (1)$$

$$CAH_2 + O_2 \rightarrow CAH^{\cdot} + OH_2^{\cdot} \quad (2)$$

$$HO_2^{\cdot} \rightleftharpoons O_2^{\cdot-} + H^+ \quad (3)$$

$$2O_2^{\cdot} + 2H^+ \xrightarrow[\text{dismutase}]{\text{superoxide}} H_2O_2 + O_2 \quad (4)$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (5)$$

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + OH^{\cdot} + OH^- \quad (6)$$

$$Fe(III) + O_2^{\cdot -} + Fe(II) + O_2 \quad (7)$$

$$H_2O_2 + O_2^{\cdot -} \rightarrow OH^{\cdot} + OH^- + O_2, \quad (8)$$

where CA = carminic acid; CAH_2 = quinone-ring-reduced carminic acid; and $CAH^{\cdot}$ = carminic acid semiquinone.

The Haber–Weiss reaction (8), frequently quoted as a source of $OH^{\cdot}$, has recently been shown to be quite slow ($k < 0.3\ M^{-1}\ sec^{-1}$) and therefore is unlikely to compete with reaction (4) (*13–15*). It has been suggested rather that *in vivo* the $OH^{\cdot}$ radical is produced by reaction of $O_2^{\cdot}$ with Fe(III) complexed with protein or ATP (*16*). In our system traces of Fe(II) could very well catalyze the production of $OH^{\cdot}$ by the analogous reactions (6) and (7).

With regard to the relative extents of inhibition of DNA cleavage shown in Fig. 3, if H_2O_2 and $O_2^{\cdot -}$ are involved in $OH^{\cdot}$ generation and if $OH^{\cdot}$ is responsible for DNA scission, then catalase and benzoate should protect to approximately equal extents. This is found to be the case. The situation with superoxide dismutase is less clear. Since this enzyme catalyzes the production of H_2O_2 in reaction (4), one might have expected the enzyme to augment the nicking. However, removal by superoxide dismutase of $O_2^{\cdot -}$ required in step (7) to reduce Fe(III) would at the same time restrict $OH^{\cdot}$ generation in step (6). The lower extent of protection by superoxide dismutase relative to catalase and benzoate may therefore represent a compromise between these conflicting requirements. Alternatively, as pointed out by a referee, the commercial Cu–Zn enzyme has been known to be inactivated by H_2O_2 which would limit its protecting function in the present application (*26*).

Carminic acid was examined by polarography in order to obtain additional confirmation of the redox processes and to quantify them by determining accurate redox potentials. The DC polarogram of $2.0 \times 10^{-4}\ M$ carminic acid at 37.6°C, with a 2-sec drop time and with KCl supporting electrolyte was determined in the pH range 4.5–9.0. It showed a single well-defined wave with $E_{\frac{1}{2}} = -0.735 \pm 0.003$ V against SCE at pH 7.0. The hydrogen ion dependence was 61.2 mV/pH unit, in good agreement with the 61.6 mV/pH expected at 37.5°C. This wave is due to the reversible reduction of the quinone moiety in a ($2e$, $2H^+$) process, which was confirmed by controlled potential coulometry. The value of $E_{\frac{1}{2}}$ is in agreement with the $E_{\frac{1}{2}}$ of −0.73 V reported by Furman and Stone (*17*); however, the initial split into two waves reported by these authors for a commercial sample in 5% ethanol was not observed.

The proposed mechanism by which reduced carminic acid cleaves DNA is common with that found for comparable conditions with daunomycin (*18*), adriamycin (*18*), mitomycin C (*19*), bleomycin (*20*), and streptonigrin (*21*). Carminic acid differs from the anthracyclines, however, in showing no evidence of binding to DNA in three

separate experiments: (i) There is no spectral change in the carminic acid chromophore upon addition of aliquots of calf thymus DNA; (ii) there is no change in the T_m of calf thymus DNA with carminic acid; and (iii) there is no relaxation of the supercoiling of PM2-CCC-DNA upon treatment with carminic acid monitored by calf thymus topoisomerase (*18*). The fact that daunorubicin (*20*) and adriamycin produce more rapid DNA strand scission than carminic acid under comparable conditions of prereduction and on a molar basis may therefore be largely attributed to the assistance of intercalative binding afforded with the anthracyclines.

With regard to the binding properties, since it was conceivable that the carboxylate anion group in the carminic acid was preventing binding by electrostatic repulsion of the phosphate residues, the methyl ester was prepared. Treatment of carminic acid with excess of diazomethane gave methyl tetra-*O*-methyl carminate **1b**. In contrast to the parent acid, **1b** showed evidence of weak binding to calf thymus DNA by a reproducible 4° decrease in the T_m value. This behavior may be contrasted with the +15.5°C increase in the T_m caused by daunomycin (*22*), which considerably stabilizes helix DNA, and may indicate weak preferential binding of **1b** to the coil form (*23*).

Reduction of **1b** in the presence of PM2-CCC-DNA produced no nicking, in accord with the previous observation in the anthracycline series that the scission process requires two adjacent hydroquinone moieties (*18*, *24*). The dc polarogram of methyl tetra-*O*-methyl carminate **1b** gave a single irreversible wave of the same diffusion current as carminic acid, but more anodic ($E_{\frac{1}{2}}$, -0.56 ± 0.010 V). Since this shift was closely similar to that found in the ring B methylated form of daunomycinone (*24*), differential pulse polarography was used to search for two processes of similar potential. Only a single symmetrical peak indicating a single process could be observed, in contrast to the two observed in daunomycinone. The anodic shift of some 130 mV, however, is similar to that observed in daunomycinone analogs (*24*) and in anthraquinones (*25*), and is due to the intramolecular hydrogen bonding possible in carminic acid and absent in the methyl ether **1b**.

In conclusion, the marked effects of carminic acid reduced *in situ* on DNA suggests a possible sensitive cell target which may well be of significance in its anticancer activity.

ACKNOWLEDGMENTS

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REFERENCES

1. J. C. Overeem and G. J. M. van der Kerk, *Rec. Trav. Chim. Pays Bas.* **83**, 1023 (1964).
2. N. Mihail and C. Craciun, *Naturwissenschaften* **57**, 500 (1970).
3. U. Sankawa, Y. Ebizuka, T. Miyazaki, Y. Isomura, H. Otsuka, S. Shibata, M. Inomata, and F. Fukuoka, *Chem. Pharm. Bull.* **25**, 2392 (1977).

4. A. DIMARCO, F. ARCAMONE, AND F. ZUNINO, "Antibiotics III—Mechanism of Action of Antimicrobial Agents and Antitumor Agents" (J. W. Corcoran and F. E. Hahn, Eds.), pp. 101–128. Springer-Verlag, New York, 1975, and references therein.
5. E. SCHUNCK AND L. MARCHLEWSKI, *Chem. Ber.* **27**, 2979 (1894).
6. M. A. ALI AND L. J. HAYNES, *J. Chem. Soc.* 1033 (1959).
7. A. R. MORGAN AND D. E. PULLEYBLANK, *Biochem. Biophys. Res. Commun.* **61**, 396 (1974).
8. G. M. RAO, A. BEGLEITER, J. W. LOWN, AND J. A. PLAMBECK, *J. Electrochem. Soc.* **124**, 199 (1977).
9. M. DIZDAROGLU, C. VON SONNTAG, AND D. SCHULTE-FROHLINDE, *J. Amer. Chem. Soc.* **97**, 2277 (1975).
10. E. G. JANZEN, *Accounts Chem. Res.* **4**, 31 (1972).
11. J. R. HARBOUR, V. CHOW, AND J. R. BOLTON, *Canad. J. Chem.* **52**, 3549 (1974).
12. F. P. SARGENT AND E. M. GARDY, *Canad. J. Chem.* **54**, 275 (1976).
13. J. J. VAN HEMMEN AND W. J. A. MEULING, *Arch. Biochem. Biophys.* **182**, 743 (1977).
14. G. J. MCCLUNE AND J. A. FEE, *FEBS Lett.* **67**, 294 (1976).
15. B. HALLIWELL, *FEBS Lett.* **72**, 8 (1976).
16. G. CZAPSKI AND Y. A. ILAN, Abstract C-11, International Conference on Singlet Oxygen and Related Species in Chemistry and Biology, August 21–26, Pinawa, Manitoba (1977).
17. N. H. FURMAN AND F. G. STONE, *J. Amer. Chem. Soc.* **70**, 3055 (1948).
18. J. W. LOWN, S. K. SIM, K. C. MAJUMDAR, AND R. Y. CHANG, *Biochem. Biophys. Res. Commun.* **76**, 705 (1977).
19. J. W. LOWN, A. BEGLEITER, D. JOHNSON, AND A. R. MORGAN, *Canad. J. Biochem.* **54**, 110 (1976).
20. J. W. LOWN AND S. K. SIM, *Biochem. Biophys. Res. Commun.* **77**, 1150 (1977).
21. R. CONE, S. K. HASAN, J. W. LOWN, AND A. R. MORGAN, *Canad. J. Biochem.* **54**, 219 (1976).
22. G. TONG, W. W. LEE, D. R. BLACK, AND D. W. HENRY, *J. Med. Chem.* **19**, 395 (1976).
23. J. D. MCGHEE, *Biopolymers* **15**, 1345 (1976).
24. G. M. RAO, J. W. LOWN, AND J. A. PLAMBECK, *J. Electrochem. Soc.* **125**, 534 (1978).
25. L. STÁRKA, L. VYSTRČIL, AND B. STÁRKOVÁ, *Chem. Listy* **51**, 1440 (1957).
26. E. K. HODGSON AND I. FRIDOVICH, *Biochemistry* **14**, 5299 (1975).