(17) D. A. Brodie, C. L. Tate, and K. F. Hooke, *Science*, **170**, 183 (1970).
(18) J. R. J. Sorenson, *J. Pharm. Pharmacol.*, **29**, 450 (1977).
(19) A. A. Alich and L. E. Wittmers, Jr., *J. Pharm. Sci.*, **69**, 725 (1980).
(20) B. Huff, Ed., "Physicians Desk Reference," 35th ed., Litton Industries, Oradell, N.J., 1981, p. 735.
(21) P. H. Guth and G. Paulsen, *Digestion*, **19**, 93 (1979).
(22) P. G. Robins, *Br. J. Exp. Pathol.*, **61**, 497 (1980).
(23) G. Wilhelmi and R. Manasse-Gdynia, *Pharmacology*, **8**, 321 (1972).
(24) J. W. D. McDonald in "Acetylsalicylic Acid: New Uses for an Old Drug," Raven, New York, N.Y., 1982, pp. 87–94.

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Hemolysis Caused by Cetomacrogol 1000: Evidence for Hydroxyl Radical Participation

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Abstract □ The mechanism of cetomacrogol 1000-induced hemolysis was investigated. Previous conclusions that peroxides are involved in the hemolytic process were confirmed. The possibility that hydrogen peroxide, superoxide, hydroxyl radical, or singlet oxygen, which are known to induce hemolysis, are involved in cetomacrogol 1000-induced hemolysis was tested by using specific inhibitors and inactivators. The hydroxyl radical ($OH^{\cdot}$) was shown to be the only apparent oxygen species involved in cetomacrogol 1000-induced hemolysis. Its contribution to the hemolytic potency of the surfactant is ~30%.

Keyphrases □ Cetomacrogol 1000—induction of hemolysis, peroxidation, hydroxyl radical participation, erythrocytes □ Surfactants—cetomacrogol 1000-induced hemolysis, peroxidation, hydroxyl radical participation □ Peroxides—role in surfactant-induced hemolysis, cetomacrogol 1000, hydroxyl radical participation

Polyoxyethylene-derived surfactants are widely used as pharmaceutical aids, although their tendency to form peroxides is well known (1–4) and their deleterious effects on various drugs have been proven (5–7). Like other surface-active compounds, the polyoxyethylene surfactants cause hemolysis when they come in contact with red blood cells (7–9). Results recently published (10) indicate that the hemolytic activity of polyoxyethylene surfactants may be ascribed to their tendency to form peroxides. It should, however, be emphasized that hemolysis is only one form of cytotoxicity.

Peroxidation is believed to be one of the main mechanisms that give rise to membrane damage and cellular death (11–16). Destruction of normal tissue as a consequence of inflammatory reactions is also ascribed to oxygen radicals. The superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide, the hydroxyl radical ($OH^{\cdot}$), and the oxygen radicals ($^1O_2$) are highly reactive and can damage most types of cellular polymers. They have been shown to oxidize proteins, peroxidize fatty acids, and cleave polysaccharides *in vitro* (15). All were found to induce lysis of various types of cells (17–19). The abundance of polyunsaturated fatty acids in cell membranes and the facility with which these undergo oxidation are assumed to be the cause of peroxide-induced lysis (20).

Because of the widespread use of these surfactants and the awareness of the pernicious effect of free radicals and especially peroxides, we decided to study the mechanism by which these compounds induce peroxidation, with the ultimate aim of finding potential antidotes to these harmful effects. Their simple structure, easy availability, and the facility with which cell damage can be estimated by means of hemolysis make erythrocytes the ideal model for such research. Cetomacrogol 1000 (polyethylene glycol 1000 monocetyl ether) was chosen as the model polyoxyethylene surfactant in this research.

EXPERIMENTAL

Materials—Cetomacrogol 1000, polyethythelene glycol 1000 monocetyl ether[1], is a solid and, thus, not sensitive to autooxidation (4). Solutions of cetomacrogol 1000 were freshly prepared. Histidine[2], tryptophan[3], mannitol[3], thiourea[4], and digitonin[2] were commercial samples. Hydroquinone[5] was crystallized from benzene prior to use. Catalase[6] was dialyzed against water (specific activity 1.57×10^6 U/ml), superoxide dismutase[3] had a specific activity of 2650 U/mg of protein. Saponin-A was obtained from *Styrax officinalis* (21).

Determination of Hemolytic Activities—All experiments were performed on citrated blood freshly drawn from albino rats. The erythrocytes, freed of plasma by three washings in cold isotonic saline, were diluted with isotonic buffer (3.95 g of $Na_2HPO_4 \cdot 2H_2O$, 0.76 g of KH_2PO_4, 7.2 g of NaCl, *q.s.* to 1000 ml with water; pH adjusted to 7.4) to give a 1% suspension. Varying quantities of hemolysin, dissolved in buffer, were added to 2 ml of the erythrocyte suspension, and the volume was increased to 4 ml with buffer. The components were added in the following order: erythrocyte suspension, buffer, and the hemolyzing agent. The mixtures were incubated for 60 min at 37° in a shaking water bath, and then were centrifuged at $1000 \times g$; the absorbance of the supernatant was determined at 540 nm. The percentage of hemolysis was determined by comparison with a sample in which 100% hemolysis was obtained by the addition of digitonin.

Effect of Inhibitors on Surfactant-Induced Hemolysis—Two sets

[1] Cetomacrogol 1000, A.B.M. Chemical Ltd., England.
[2] E. Merck, Darmstadt, West Germany.
[3] Sigma Chemical Co., St. Lous, Mo.
[4] Riedel-De Haen AG. Seelze-Hannover.
[5] Photographic grade; May and Baker Ltd., Dagenham, England.
[6] Sigma—1000.

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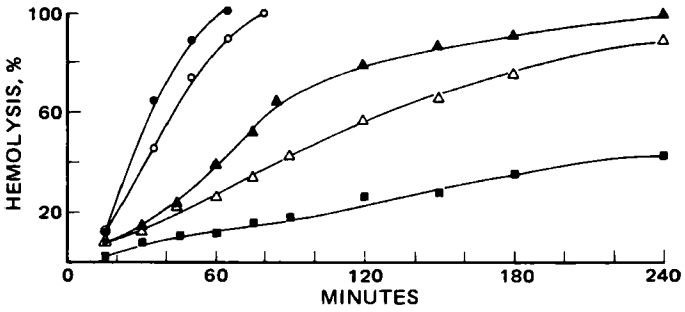

Figure 1—*Time dependence of cetomacrogol 1000-induced hemolysis. Cetomacrogol 1000 concentration: (●) 1.5 × 10⁻⁵ M; (○) 1.3 × 10⁻⁵ M; (▲) 9.5 × 10⁻⁶ M; (△) 7.6 × 10⁻⁶ M; (■) 5.7 × 10⁻⁶ M.*

of experiments were run in parallel: (*a*) a regular hemolysis test and (*b*) the same incubation mixture as in (*a*) but with inhibitor solutions (in buffer) substituting for part of the buffer. The components were added as follows: erythrocytes, buffer, inhibitor, hemolysin. Percentage inhibition was determined as:

$$100 \times \left(1 - \frac{\%\text{ hemolysis with inhibitor}}{\%\text{ hemolysis without inhibitor}}\right)$$

Effect of Visible Light on Cetomacrogol 1000-Induced Hemolysis—Equal volumes of mixtures consisting of erythrocytes, buffer, and cetomacrogol 1000 were introduced into eight test tubes. Four of the test tubes were shielded from light by covering them with aluminium foil. All eight of the test tubes were incubated for 1 hr at 37° and processed as described above.

RESULTS

Figure 1 shows the cetomacrogol 1000-induced hemolysis at various time intervals. The five curves represent different cetomacrogol 1000 concentrations; their shape is in accordance with previous observations that hemolysis produced by polyoxyethylene cetostearyl ethers progresses slowly toward 100% irrespective of the surfactant concentration (10).

To ensure reproducibility of the results and to provide a common basis for comparison of results of different experiments, the extent of the hemolysis was tested after exactly 1 h of incubation at 37°. The percentage of hemolysis was, therefore, reproducible to within 3% hemolysis.

No information is available on the identity of the active tissue-damaging radical produced during autooxidation of the polyoxyethylene surfactants, especially when they are in contact with living cells. We therefore decided to test the inhibitory influence of a variety of enzymes and chemicals which inactivate specific peroxides and radicals known to induce hemolysis. The effectiveness of the various inhibitors was tested at three cetomacrogol 1000 concentrations: 1.3×10^{-5} *M*, 1×10^{-5} *M*, and 7.6×10^{-6} *M*. These concentrations normally cause ~80, 50, and 30% hemolysis within 1 hr.

Catalase—Hydrogen peroxide either by itself or as a precursor of other active radical species is a well-known inducer of tissue damage and hemolysis (20, 22). The enzyme catalase, which catalyzes the conversion of H_2O_2 to O_2 and H_2O, was tested as a potential inhibitor for cetomacrogol 1000-induced hemolysis. The results (Table I) show that catalase had no inhibitory effect on cetomacrogol 1000-induced hemolysis even at levels as high as 625 U/ml.

Superoxide Dismutase—Superoxide radicals (O_2^-), generated enzymatically or photochemically, have been shown to induce lipid peroxidation, to damage membranes, and to kill cells (17, 20). The enzyme superoxide dismutase, which gives rise to the dismutation $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (23), was used to test the possibility of O_2^- being the active hemolyzing agent. No inhibition was obtained with superoxide dismutase (65 U/ml) nor with a superoxide dismutase–catalase mixture. All results are summarized in Table I.

Oxygen Radicals ($^1O_2$)—Hemolysis can be the result of the photosensitized production of $^1O_2$ using either endogenous or exogenous photosensitizers (19). The possibility that singlet oxygen was responsible for the cetomacrogol 1000-induced hemolysis was evaluated by adding specific $^1O_2$ quenchers, *i.e.*, the aromatic amino acids histidine and tryptophan, and hydroquinone (15, 18, 20). The three inhibitors were tested at the highest possible concentrations: hydroquinone at concentrations $>2.5 \times 10^{-4}$ caused damage to the erythrocytes (they turned brown); the maximal concentrations for histidine and tryptophan were those allowed by their solubility.

The results of these experiments are summarized in Table I. Of the three $^1O_2$ quenchers, only histidine caused slight inhibition of hemolysis, while tryptophan and hydroquinone produced no effect at all.

Hydroxyl Radicals—These radicals are very reactive and can interact with unsaturated fatty acids, which are components of the cell membrane, either through hydrogen abstraction or by addition to the unsaturated system (24). Among the oxidizing radicals the hydroxyl radical is the most powerful and most deleterious to the living membrane (25). The role of the hydroxyl radical in the hemolytic effect of cetomacrogol 1000 was tested by the addition of the specific hydroxyl radical quenchers, mannitol and thiourea. Both produced significant inhibition. Inhibition as function of mannitol and thiourea concentration is represented in Figs. 2 and 3, respectively. At high levels of hemolysis, an almost linear correlation between inhibitor concentration and inhibitor effect is observed, whereas at low levels, inhibition increases with inhibitor concentration until a plateau is obtained. In no case does inhibition exceed 35%. The possibility that the inhibition might be due to some nonspecific protective effect of the inhibitors on the red blood cell membranes was eliminated by testing their effect on saponin hemolysis. Digitonin and *Styrax* saponin, two highly potent crystalline compounds, were chosen to represent

Table I—Effect of Catalase, Superoxide Dismutase, Hydroquinone, Tryptophan, and Histidine on Cetomacrogol 1000-Induced Hemolysis

Cetomacrogol 1000 Concentration, *M*	Hemolysis, %	Inhibitor	Inhibitor Concentration	Inhibition, %
1.3×10^{-5}	80 ± 0.2 (5)	Superoxide dismutase	66.2 μg/ml	—
1×10^{-5}	50 ± 0.3 (5)		66.2 μg/ml	—
1.3×10^{-5}	80 ± 0.2 (5)	Catalase	250 μg/ml	—
			625 μg/ml	—
1×10^{-5}	50 ± 0.3 (5)		250 μg/ml	—
			625 μg/ml	—
1.3×10^{-5}	82 ± 0.5 (5)	Hydroquinone	2.2×10^{-4} *M*	—
			2.2×10^{-5} *M*	—
			2.2×10^{-6} *M*	—
1×10^{-5}	51 ± 0 (5)		2.2×10^{-4} *M*	—
1.3×10^{-5}	82 ± 0.5 (5)	Tryptophan	1.3×10^{-2} *M*	—
1×10^{-5}	51 ± 0 (5)		1.3×10^{-2} *M*	—
1.3×10^{-6}	82 ± 0.5 (5)	Histidine	5×10^{-2} *M*	7 ± 1.8 (5)
			2×10^{-2} *M*	7 ± 1.7 (5)
			1×10^{-2} *M*	—
			5×10^{-3} *M*	—
1×10^{-5}	51 ± 0 (5)		5×10^{-2} *M*	16 ± 1.1 (5)
			2×10^{-2} *M*	10 ± 1.5 (5)
			1×10^{-2} *M*	—
			5×10^{-3} *M*	—
7.6 ± 10^{-6}	31 ± 1.2 (5)		5×10^{-2} *M*	21 ± 0.9 (5)
			2×10^{-2} *M*	10 ± 1.2 (5)
			1×10^{-2} *M*	—

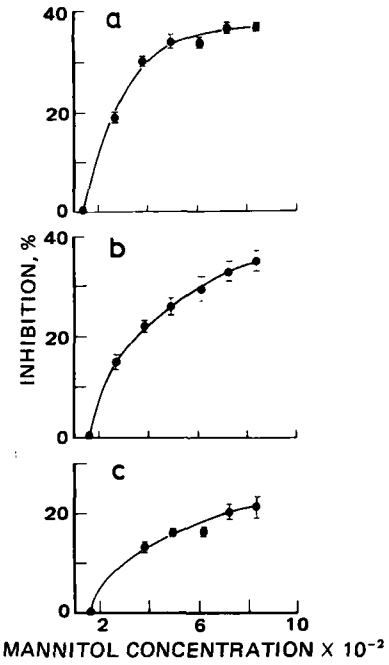

Figure 2—*Inhibition of cetomacrogol 1000-induced hemolysis as a function of mannitol concentration. Key: (a) cetomacrogol 1000, 7.6 × 10^{-6} M; (b) cetomacrogol 1000, 1 × 10^{-5} M; (c) cetomacrol 1000, 1.3 × 10^{-5} M.*

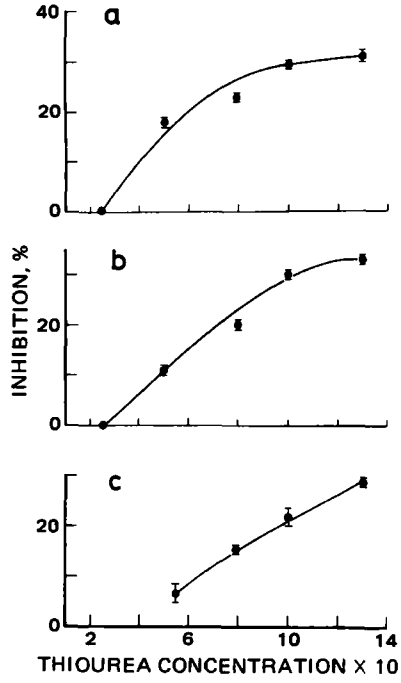

Figure 3—*Inhibition of cetomacrogol 1000-induced hemolysis as a function of thiourea concentration. (a) cetomacrogol 1000, 7.6 × 10^{-6} M; (b) cetomacrogol 1000, 1 × 10^{-5} M; (c) cetomacrogol 1000, 1.3 × 10^{-5} M.*

the saponins. The experiments were performed at two levels of hemolysis (*i.e.*, saponin concentrations). Mannitol and histidine had no effect on saponin hemolysis. Thiourea produced some inhibition at high inhibitor concentrations. However at 1.3 × 10^{-3} *M*, a concentration at which maximal inhibition of cetomacrogol 1000-induced hemolysis was obtained, no effect was observed (Table II).

Free radical reactions are often light catalyzed. We therefore tested whether cetomacrogol 1000-induced hemolysis is promoted by visible light. The experiments were performed at four different surfactant concentrations, and the results are summarized in Table III. They clearly show that the extent of hemolysis is indeed enhanced under the influence of light.

DISCUSSION

The results of the present investigation confirm previous conclusions (10) that the hemolytic activity of polyoxyethylene-derived surface-active agents is to be ascribed, at least in part, to their tendency to form peroxides. Various oxygen radical species and peroxides are known to induce hemolysis as a result of lipid peroxidation (12, 13, 15, 20). Our experiments indicate that in cetomacrogol 1000-induced hemolysis, the hydroxyl radical is strongly involved in the process.

The polyoxyethylene surfactants are polyethers and, therefore, their spontaneous autocatalytic peroxidation has been described as taking place *via* a free radical chain reaction as follows (3):

$$RH + \text{light or catalyst} \rightarrow R\cdot + H\cdot$$

Scheme I—Initiation

$$R\cdot\, O_2 \rightarrow ROO\cdot$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

Scheme II—Propagation

Free radicals also may be formed by the processes in Scheme III, and removed by those in Scheme IV:

$$ROOH \rightarrow ROO\cdot + H\cdot$$

$$ROOH \rightarrow RO\cdot + \cdot OH$$

$$2ROOH \rightarrow ROO\cdot + RO\cdot + H_2O$$

Scheme III

Table II—Effect of Histidine, Mannitol, and Thiourea on Saponin Hemolysis

Saponin	Saponin Concentration, *M*	Hemolysis, %	Inhibitor	Inhibitor Concentration	Inhibition, %
Styrax saponin	8×10^{-7}	62 ± 0 (5)	Histidine	5×10^{-2}	0 (5)
	6×10^{-7}	46 ± 1 (4)			0 (4)
Digitonin	1.3×10^{-6}	42 ± 1 (5)			0 (5)
	1.0×10^{-6}	25 ± 1 (4)			0 (4)
Styrax saponin	8×10^{-7}	62 ± 0 (5)	Mannitol	1×10^{-1}	0 (5)
	6×10^{-7}	46 ± 1 (4)			0 (4)
Digitonin	1.3×10^{-6}	42 ± 1 (5)			0 (5)
	1.0×10^{-6}	25 ± 1 (4)			0 (4)
Styrax saponin	8×10^{-7}	62 ± 0 (5)	Thiourea	1.3×10^{-1}	48 ± 0 (5)
				6.5×10^{-2}	20 ± 0.3 (5)
				1.3×10^{-2}	0 (5)
	6×10^{-7}	46 ± 1 (4)		6.5×10^{-2}	47 ± 1 (4)
				1.3×10^{-2}	0 (4)
Digitonin	1.3×10^{-6}	42 ± 1 (5)		1.3×10^{-1}	50 ± 1.3 (5)
				6.5×10^{-2}	40 ± 1.5 (5)
				1.3×10^{-2}	0 (5)
	1.5×10^{-6}	58 ± 2 (4)		6.5×10^{-2}	30 ± 1 (4)
				1.3×10^{-2}	0 (4)

Table III—Effect of Visible Light on Cetomacrogol 1000-Induced Hemolysis

Cetomacrogol 1000 Concentration, *M*	Hemolysis in Light, %	Hemolysis in Darkness, %	Inhibition of Hemolysis in Darkness, %
1.3×10^{-5}	80 ± 2 (4)	66 ± 3 (4)	20
1.0 ± 10^{-5}	50 ± 1 (4)	37 ± 2 (4)	26
7.6 ± 10^{-6}	24 ± 2 (4)	18 ± 0.5 (4)	25
5.7 ± 10^{-6}	10 ± 0.5 (4)	8 ± 0.5 (4)	20

$$2RO_2^{\cdot} \xrightarrow{(a)} \text{inactive products}$$

$$RO_2^{\cdot} + R^{\cdot} \xrightarrow{(b)} \text{inactive products}$$

$$2R^{\cdot} \xrightarrow{(c)} \text{inactive products}$$

Scheme IV—Termination

This indicates that hydroxyl radicals may in fact be produced during polyoxyethylene surfactant autooxidation, as suggested by our findings on the active hemolyzing factor. The observation that visible light enhances cetomacrogol 1000-induced hemolysis further substantiates this suggestion.

The concentrations of inhibitors used in the present investigation were high when compared with those used by Kellog and Fridovich (20) to inhibit the hemolysis obtained by xanthine oxidase–acetaldehyde. These authors however used a much lower erythrocyte concentration for their experiments. This difference in the experimental conditions no doubt affects the extent of hemolysis as well as the extent of inhibition obtained by inhibitors.

The shapes of the curves representing the extent of inhibition by mannitol and thiourea as a function of inhibitor concentration are peculiar (Figs. 2 and 3). Although inhibition increases with inhibitor concentration, the curves reach a plateau at ~30% inhibition. The reason for the incomplete inhibition by $OH^{\cdot}$ scavengers may be due to other radicals obtained in the course of the surfactant autooxidation (see Schemes I–IV).

The possibility that histidine might scavenge such radicals seems controvertible since a mixture of histidine–mannitol did not enhance the inhibition beyond that of mannitol alone[7]. The assumption that other peroxide radicals take part in the hemolytic process also seems questionable in view of the fact that experiments conducted under a nitrogen atmosphere also produced only 30% inhibition (10). The high degree of consistency of these results suggests that although surface activity of the polyoxyethylene-derived surfactants could not be correlated with their hemolytic potency, the solubilizing properties of the surfactant contribute to the hemolytic process. Our present experiments suggest that the contribution of the surface activity factor to cetomacrogol 1000-induced hemolysis is ~70%, while the contribution of the $OH^{\cdot}$ radicals is ~30%.

[7] Unpublished results.

REFERENCES

(1) R. Hamburger, E. Azaz, and M. Donbrow, *Pharm. Acta Helv.* **50,** 10 (1975).

(2) J. W. McGinity, J. A. Hill, and A. L. La Via, *J. Pharm. Sci.*, **64,** 356 (1975).

(3) M. Donbrow, E. Azaz, and A. Pillersdorf, *J. Pharm. Sci.*, **67,** 1676 (1978).

(4) M. Donbrow, R. Hamburger, and E. Azaz, *J. Pharm. Pharmacol.*, **27,** 160 (1975).

(5) H. N. Glassman, *Science*, **111,** 688 (1950).

(6) E. Azaz, M. Donbrow, and R. Hamburger, *Pharm. J.*, **211,** 15 (1973).

(7) E. Azaz and R. Segal, *J. Pharm. Pharmacol.* **29,** 322 (1977).

(8) T. Kondo and M. Tomiza *J. Pharm. Sci.*, **57,** 1246 (1968).

(9) B. Y. Zaslavsky, N. N. Ossipov, V. S. Krivich, L. P. Baholdina, and S. V. Rogozhin, *Biochim. Biophys. Acta*, **507,** 1 (1978).

(10) E. Azaz, R. Segal, and I. Milo-Goldzweig, *Biochim. Biophys. Acta*, **646,** 444 (1981).

(11) P. M. Pfeifer and P. B. McCay, *J. Biol. Chem.*, **246,** 6401 (1971).

(12) G. L. Plaa and H. Witschi, *Ann. Rev. Pharmacol. Toxicol.*, **16,** 125 (1976).

(13) T. L. Dormandy, *Lancet*, **i,** 647 (1978).

(14) A. Bendetti, A. F. Casini, M. Ferraly, and M. Comporti, *Biochem. J.*, **180,** 303 (1979).

(15) R. H. Simon, C. H. Scoggin, and D. Patterson, *J. Biol. Chem.*, **256,** 7181 (1981).

(16) M. Kunimoto, K. Inoue, and S. Nojima, *Biochim. Biophys. Acta*, **646,** 169 (1981).

(17) L. Simchovitz and J. Spilberg, *Immunology*, **37,** 301 (1979).

(18) R. Nilsson, G. Swanbeck, and G. Wennersten, *Photochem. Photobiol.*, **22,** 183 (1975).

(19) N. I. Krinski, in "Singlet Oxygen" H. H. Wasserman and R. W. Murray, Eds., Academic New York, N.Y., 1979, p. 597.

(20) E. W. Kellog and I. Fridovich, *J. Biol. Chem.*, **252,** 6721 (1977).

(21) R. Segal, H. Govrin and D. V. Zaitschek, *Tetrahedron Lett.*, **1964,** 527.

(22) M. Naim, B. Gestetner, A. Bondi, and Y. Birk, *J. Agric. Food Chem*, **24,** 11,174 (1976).

(23) I. Fridovich, *Science*, **201,** 875 (1978).

(24) D. C. Nonhebel and J. C. Walton," Free Radical Chemistry," Cambridge University Press, England, 1974, p. 358.

(25) H. A. O. Hill, in "Oxygen Free Radicals and Tissue Damage," Ciba Foundation Symposium 65 Experta Medica, Amsterdam, 1979, p. 5.