Volume 3 no.5 May 1976 Nucleic Acids Research

The mechanism of DNA strand breakage by vitamin C and superoxide and the protective roles of catalase and superoxide dismutase

A. Richard Morgan, Richard L. Cone and Terry M. Elgert

Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada

Received 18 August 1975

ABSTRACT

Vitamin C breaks DNA only in the presence of oxygen. Superoxide dismutase has no effect on the reaction but catalase suppresses it. Superoxide also gives rise to breaks in DNA suppressible by both superoxide dismutase and catalase. The hydroxyl radical seems to be the agent responsible for strand cleavage itself.

INTRODUCTION

Vitamin C has received a great deal of popular attention recently primarily as a result of the claims of Pauling[1] that very large doses are beneficial in combatting the common cold. The controversy that has arisen since, suggests a lack of good data and the possibility of large individual variabilities in response to the vitamin therapy. Yew[2] has shown that guinea pigs thrive best on diets in which ascorbate is about 40 times the recommended dose on a body-weight basis. There was also a particularly marked variation from animal to animal at the lower levels of intake. The chemistry of vitamin C is complex and a multiplicity of roles is expected *in vivo*. It is required as a cofactor in some oxidation reactions requiring molecular oxygen, for example the oxidation of dopamine to noradrenalin or homogentisate to 4-maleylacetoacetate. It accelerates hydrolytic deamidation of certain peptides and this has been implicated as a molecular "timer" in biological systems.[3] It has also been shown to be an anti-viral agent against certain lactobacillus bacteriophages.[4]

Various reducing agents[5], including vitamin C[6,7] have been reported to cleave DNA and in the case of vitamin C the cleavage was shown to be oxygen dependent. We independently made similar observations using an assay which we have recently developed for measuring the conversion of covalently-closed circular (ccc) DNA to open circular (oc) DNA.[28] The assay is both rapid and sensitive exploiting the fluorescence of ethidium bound to duplex DNA, and encouraged us to investigate the mechanism of DNA and RNA strand cleavage.

In a separate study on the antiviral properties of vitamin C[8], the viability of bacteriophage R17 was decreased by 10^6 in four minutes in 1 mM vitamin C, 10 μM Cu^{++}. Under these conditions the RNA is extensively degraded but the phage particle was essentially intact and could still inject the degraded RNA into the E. Coli host, (Wong and Paranchych, private communication). It therefore appears that the lethal event is at the level of RNA degradation. We attempted to extend the impressive antiviral activity of vitamin C to testing the effects of vitamin C ingestion on the capacity for blood serum to inactivate R17 phage. Serum completely protected R17 phage against inactivation even with exogenously added vitamin C. The protection was shown to be heat-labile and was traced to catalase.

In parallel studies the requirement for oxygen suggested the possibility of the superoxide radical being involved. Superoxide is formed by the donation of an electron to O_2 and since vitamin C forms a stable free radical with the loss of an electron, it could provide a source of electrons. In addition it was found that superoxide did indeed lead to DNA strand cleavage which was completely inhibited by catalase. An enzyme which dismutates superoxide to oxygen and hydrogen peroxide (superoxide dismutase) has been characterized by McCord and Fridovich[9] and shown to be ubiquitous in aerobic organisms. The importance of superoxide dismutase is indicated by the fact that obligate anaerobes do not contain superoxide dismutase and are killed in air, suggesting a crucial role for the enzyme in the evolution of life as atmospheric oxygen built up. The cleavage of DNA induced by the superoxide radical was completely suppressed by even nanomolar levels of superoxide dismutase, as well as catalase. However the vitamin C induced cleavage was unaffected by superoxide dismutase even at 1000 times the level used for suppression of superoxide cleavage. It seemed unlikely that the hypothesized mechanism of vitamin C giving up an electron to oxygen was correct. Instead, as will now be shown, it appears that vitamin C and superoxide can both give rise to hydroxyl radicals generated from hydrogen peroxide produced in the reactions and it is the hydroxyl radical which is directly responsible for DNA strand scission.

ASSAY FOR DNA STRAND BREAKAGE

The assay exploits the enhancement of ethidium fluorescence when it intercalates duplex DNA and the renaturability of ccc DNA after heat denaturation at pH 12.[28] The details of the assay are outlined in the legend to Figure 1.

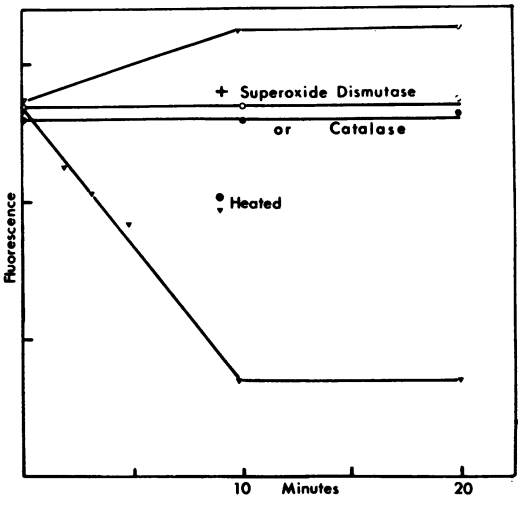

Figure 1. The cleavage of ccc PM2 DNA induced by $\cdot O_2^-$ and its inhibition by superoxide dismutase. Xanthine oxidase was assayed by following the reduction of cytochrome C at 550 nm[9]. All reactions were in 0.1 mM EDTA, 50 mM potassium phosphate pH 7.6, xanthine 50 µM and xanthine oxidase 0.12 µM. Cytochrome C at 0.1 mM showed an increase in A_{550} of 0.008 per minute at 22° and this was reduced to 0.001 in the presence of superoxide dismutase at 4 µg/ml (kindly donated by Dr. Alan Davison). ccc PM2 DNA at 0.84 A_{260} was substituted for cytochrome C, and 20 µl samples were taken at intervals and added to 2 ml of alkaline ethidium bromide mix[28]. The fluorescence readings were taken and the sample was then heated for 2 minutes at 98° to irreversibly denature any oc DNA, cooled and the fluorescence reread. The ccc PM2 DNA used contained a small amount of oc DNA that lead to a 20% loss in fluorescence after heating. This was also observed in the zero time samples in the reaction mixtures. This was subtracted from all fluorescence readings which had not been heated. Complete conversion of ccc DNA to oc DNA gives a 30% rise in fluorescence and 100% loss after heating[28]. In the absence of superoxide dismutase the ccc DNA is converted to oc DNA as shown by the increase and decrease of fluorescence before and after heating respectively, Δ——Δ and ▲——▲ . In the presence of 4 µg/ml superoxide dismutase no detectable cleavage occurred o——o and ●——●. These reactions were at 37°. Superoxide dismutase which had been denatured by heating at 98° for 10 minutes at 40 µg/ml under the reaction conditions and additionally 0.1 mg/ml gelatin had no effect on the rate of cleavage of ccc DNA by $\cdot O_2^-$ Catalase at 1.25 µg/ml also completely inhibited cleavage of ccc DNA giving essentially the same results as superoxide dismutase.

The 200 fold dilution of reaction mixture samples into the assay mixture is sufficient to prevent interference with the fluorescence assay by metal ions. The possibility of interference with the fluorescence is readily checked by adding samples to DNA in ethidium solution and observing any change in fluorescence. The main advantages of the assay are the sensitivity (sub-

microgram quantities of DNA are required) and the simplicity and speed (two readings required in a fluorometer before and after heating the assay solution). Other assays of comparable sensitivity require sedimentation of the DNA and are very time consuming.

SUPEROXIDE INDUCED CLEAVAGE OF DNA

From studies on the radiosensitivity of phage DNA in aqueous solution[10] and the protective effect of iodide as an effective hydroxyl radical scavenger, it was concluded that superoxide ($\cdot O_2^-$) does not inactivate DNA. Pulse radiolysis experiments[12] also indicated that $\cdot O_2^-$ did not react directly with DNA since the presence of DNA did not affect the rate of disappearance of $\cdot O_2^-$. However, as shown in Figure 1, $\cdot O_2^-$ generated by xanthine-xanthine oxidase[11] did convert ccc DNA to oc DNA. That it was indeed superoxide that was responsible for cleaving the DNA was confirmed by complete inhibition of cleavage in the presence of low levels of superoxide dismutase. It was further shown that heat treatment of the superoxide dismutase led to reversal of the inhibition as expected of an enzymatic reaction. Catalase also efficiently prevented $\cdot O_2^-$ cleaving DNA suggesting an indirect attack on the DNA.

The most likely mechanism is:

$$2H^+ + \cdot O_2^- + \cdot O_2^- \longrightarrow H_2O_2 + O_2^* \quad (1)$$

$$\cdot O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2 \quad [29] \quad (2)$$

$$\cdot OH + DNA \longrightarrow DNA^\cdot + H_2O \quad (3)$$

This would account for the observation that both superoxide dismutase and catalase prevent $\cdot O_2^-$ cleavage of DNA. As a further test of this postulated mechanism it was found that $\cdot OH$ scavengers inhibited the cleavage of DNA by $\cdot O_2^-$ (Table 1) and also that when low levels of H_2O_2 were added to the reaction, DNA cleavage was stimulated (Figure 2). However, at higher levels of H_2O_2 the cleavage of DNA was diminished overall, possibly by inactivating xanthine oxidase or by competing free radical decay pathways. H_2O_2 alone gives no cleavage (Figure 2). The hydroxyl radical is one of the strongest known oxidising agents and from studies on the origin of DNA lesions from radiation, hydroxyl radicals have been implicated as the main source of strand cleavage[13], presumably by attack on the C_5' or C_3' H's with production of a DNA radical

Table I Inhibition of Superoxide Induced Cleavage of PM2 DNA by Hydroxyl Radical Scavengers*

Scavenger	mM	% Inhibition of Cleavage	Scavenger	mM	% Inhibition of Cleavage
Mannitol	0.25	3	Formate	0.25	3
	1.25	18		1.25	22
	2.5	31		2.5	39
	5	47	Ethanol	0.25	6
Benzoate	0.25	13		1.25	13
	1.25	34		2.5	29
	2.5	39	DMSO	0.25	21
KI	0.25	28		1.25	59
	1.25	73		2.5	69
	2.5	86		5	83

* The assay conditions were as for Figure 1 and the % inhibition of cleavage was computed from the ratio of the % cleavage in the presence of scavenger to the % cleavage in the absence of scavenger (about 80% at 10 minutes).

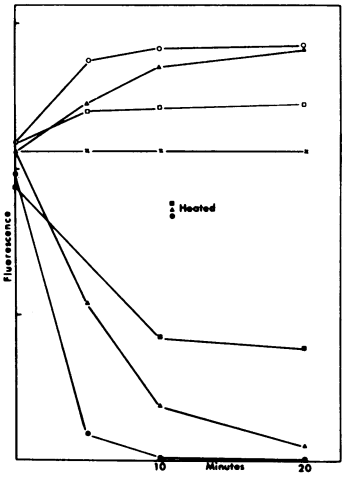

Figure 2 The effect of hydrogen peroxide on superoxide-induced cleavage of ccc DNA. The reaction conditions were those of Figure 1 with xanthine oxidase Δ——Δ, and with 0.27 mM H_2O_2 o——o or 10.6 mM H_2O_2 □——□ added in addition; H_2O_2 (1 mM) added alone in the absence of xanthine and xanthine oxide X——X, with no change in fluorescence after heating except that due to the small amount of oc DNA.

leading to phosphodiester bond breaks[14]. Hydroxyl radicals cause single-strand breaks consistent with the finding of $^{\cdot}O_2^-$ cleavage. After 40%

cleavage of PM2 ccc DNA by $\cdot O_2^-$ the product was analysed by analytical sedimentation in alkaline CsCl. A fast sedimenting band of denatured ccc DNA (86 S) comprised about 60% of the optical density(confirming the fluorescence assay) and was followed by two bands of equal intensity (25 S and 30 S) corresponding to the linear and circular single strands expected from singly-nicked DNA. In Equation (1) O_2^* corresponds to excited singlet oxygen formed during the non-enzymatic dismutation of $\cdot O_2^-$ [15]. There is no evidence that it is significantly interacting with the DNA to produce breaks although it has recently been implicated as a microbicidal agent in phagocytosis[16].

Vitamin C Induced Cleavage of DNA

The dependence of the vitamin C induced conversion of ccc DNA to oc DNA on oxygen is shown in Figure 3.

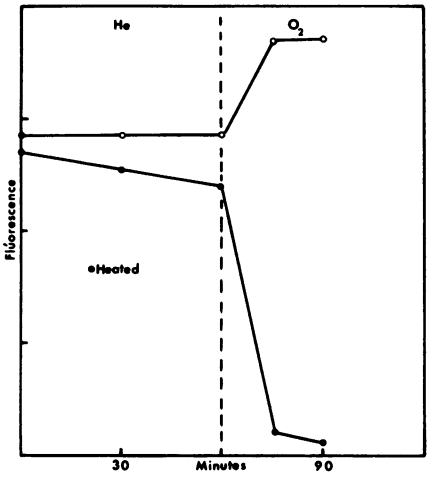

Figure 3 Cleavage of ccc DNA induced by vitamin C and oxygen. The reaction contained 20 mM Tris HCl pH 8, 1 mM vitamin C, 1 A_{260} PM2 DNA. Water was purged with helium in a glove bag for 10 minutes and then to this was added concentrated solutions of Tris HCl and DNA to give the appropriate final concentrations. After a brief further purging a solution of vitamin C made up in the deoxygenated water was added. Samples of 10 µl were added to 2 ml of ethidium assay mixture. After 1 hour pure O_2 was bubbled through the solution for 1 minute.

When the solutions are purged with helium virtually no detectable conversion occurs but the moment oxygen is bubbled into the solution ccc DNA rapidly disappears. This reaction is strongly inhibited by catalase at nanomolar levels as observed for the superoxide induced cleavage but in contrast, superoxide dismutase has no effect even at micromolar levels, a thousand times that used in the superoxide studies (Figure 4).

Cupric ions have been observed to stimulate the vitamin C cleavage reaction [7,8] as has been confirmed. Figure 5 shows that DNA is cleaved efficiently

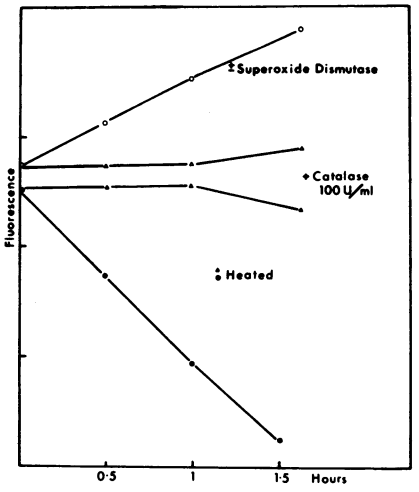

Figure 4. The inhibition of the vitamin C induced ccc DNA cleavage in the presence of catalase. The reaction mixture was as for Figure 3 except that aerated water was used for solutions. The cleavage of DNA was followed as previously, o——o and was the same even at 1 mg/ml of added superoxide dismutase. In the presence of bovine catalase (crystalline, obtained from Sigma Chemical Co.) at 1.25 µg/ml there was essentially no cleavage. The slight apparent cleavage at 1.5 hours is real and corresponds to an impurity (possibly a nuclease) in the catalase since at higher levels of catalase the DNA was shown to be cleaved in the absence of vitamin C.

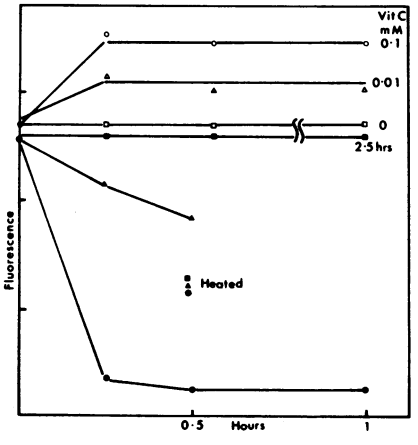

Figure 5. DNA cleavage induced by vitamin C in the presence of $CuCl_2$. The reaction mixtures contained vitamin C at the concentration indicated and 10 µM $CuCl_2$. PM2 DNA, Tris HCl and the assay procedure were as for Figure 3.

at very low levels of vitamin C in the presence of 10 μM $CuCl_2$. A possible course of events is as follows:

$$\text{Ascorbate} + O_2 \longrightarrow \text{Dehydroascorbate} + H_2O_2 \quad (4)$$

$$\text{Ascorbate} + 2\ Cu^{++} \longrightarrow \text{Dehydroascorbate} + 2\ Cu^{+} \quad (5)$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{++} + \cdot OH + OH^{-} \quad (6)$$

This would account for the inhibition by catalase and lack of any effect by superoxide dismutase as well as the stimulatory effect of cupric ions. Ascorbate will readily reduce cupric ions and in fact at higher concentrations than used in these reactions will reduce it to metallic copper. Cuprous ions react with H_2O_2 to produce hydroxyl radicals analagously to Fenton's reagent ($Fe^{++} + H_2O_2$)[17]. In control reactions it was shown that under the assay conditions cupric ions alone or H_2O_2 alone did not give rise to any detectable DNA cleavage, although together there was a very slow cleavage probably due to H_2O_2 acting as a reducing agent to convert Cu^{++} to Cu^{+} with release of O_2 as well as its more usual oxidising role. There was a very rapid reaction with Fenton's reagent, essentially no ccc DNA being present after one minute with 10^{-4} M Fe^{++} and 3% H_2O_2 in 40 mM Tris pH8 buffer. Cuprous salts are too insoluble unless generated in situ to generate hydroxyl radicals via this route. Attempts to show that the Cu^{++}/vitamin C cleavage of DNA was oxygen dependent by degassing with helium were unsuccessful. However, although under these conditions with vitamin C alone oxygen dependence could be shown, we cannot be sure traces of oxygen are not still present, which seems especially likely since the cleavage is still inhibited by catalase. Thus the probable explanation is in the presence of cupric ions the system becomes more sensitive to trace amounts of oxygen. Again the vitamin C cleavage of DNA was sensitive to agents which scavenge ·OH radicals, (Table I) as expected of reaction (6).

Radiation Damage to DNA

We have shown that catalase and superoxide dismutase will protect DNA from the degradative action of superoxide and hydroxyl radicals when the latter are generated from hydrogen peroxide. The discovery of superoxide dismutase for removing superoxide has set a precedent for searching for enzymes which might remove radicals; in particular it is conceivable that catalase may remove hydroxyl radicals themselves. Initial studies on the protection of DNA by catalase from Fenton's reagent showed that levels of catalase just sufficient to protect the DNA were also sufficient to remove all the hydrogen peroxide. In other words it was not possible to find an enzyme level that would remove hydroxyl radicals without destroying all the H_2O_2 at the same time. The radiation induced formation of hydroxyl radicals would appear to

be the best mechanism for studying the effect of catalase on hydroxyl radicals. Radiolysis of water gives rise principally to the radicals ·H, ·OH and e^-_{aq}. By purging the solutions with N_2O, not only is oxygen removed which could give rise to $\cdot O_2^-$ but also e^-_{aq} reacts with N_2O and H_2O to give N_2, OH^- and ·OH, elevating the level of hydroxyl radicals. The H· radicals react with DNA to a minor extent compared to ·OH radicals. Therefore a solution of ccc PM2 DNA (0.5 A_{260}) in 25 mM pH 7.4 phosphate buffer purged with N_2O was irradiated with a ^{60}Co source at a dose rate of 16 rads/min, with and without catalase for 15 mins. In the reaction without catalase 53% ccc DNA remained and with 1000 units/ml of catalase 60% remained. This suggests that catalase has no marked protective effect even at rather high levels against ·OH. This confirms that whereas $\cdot O_2^-$ decomposition by dismutation is relatively slow because of the negative charge (pK_a of HO_2 is 4.8) and can be enhanced by 10^5 by superoxide dismutase[11], ·OH being uncharged and a powerful oxidising agent reacts in a diffusion controlled manner, and is unlikely to be removed by any putative enzyme.

Biological and Chemical Implications

The above work stresses the crucial roles that superoxide dismutase and catalase have in protecting the hereditary apparatus from free radical damage and suggests in part why these enzymes are so ubiquitous. There are a large number of enzymic reactions giving rise to $\cdot O_2^-$ and of particular interest is the considerable flux of $\cdot O_2^-$ generated by polymorphonuclear leukocytes (granulocytes). It has been shown that in patients with chronic granulomatous disease there is about a 50 fold reduction in $\cdot O_2^-$ production[18]. Although yet to be proven this would indicate a role for superoxide in phagocytosis. This is further suggested by the fact that catalase-containing microorganisms are not phagocytosed by diseased granulocytes whereas catalase negative organisms are killed if a source of H_2O_2 is made available. This would be consistent with the mechanism postulated for DNA strand breakage by $\cdot O_2^-$ via hydroxyl radicals. Recently the degradation of hyaluronic acid by superoxide was also shown to be catalase and superoxide dismutase inhibitable[19], and it was suggested that the synovial fluid degradation in an inflamed joint was the result of superoxide production by the polymorphonuclear leukocytes. The anti-inflammatory effect of injected superoxide dismutase bears this out and clinical applications are evident. It is also of interest that polymorphonuclear leukocytes are more radiation resistant than T & B cells which probably correlates with their ability to produce $\cdot O_2^-$ and withstand the potentially lethal action.

Many reducing agents are capable of breaking down DNA and some of these data can now be interpreted as occurring via $\cdot O_2^-$ production. In particular, the action of thiol reagents is most simply explained by the recent findings that autoxidation of thiols generates superoxide[20]. Since dithiothreitol and mercaptoethanol are widely used in many enzyme preparations, the addition of superoxide dismutase would be advisable in any studies involving high molecular weight DNAs, especially if structural integrity is required as for ccc DNA. Claims that the DNA in chromatin is held together by disulfide links[21] because of its sensitivity to mercaptoethanol should also be more carefully examined in the presence of catalase and superoxide dismutase. Although with dithiothreitol at 0.1 mM there was no significant cleavage of PM2 DNA in 4 hours, addition of 0.2 mM Fe SO_4 resulted in 37% cleavage in 30 minutes, an expected stimulation[20]. Dilute solutions of SO_2 have also been found to break down DNA via a free radical mechanism[22], and it has also been shown that in the presence of EDTA the autoxidation of sulfite proceeds via $\cdot O_2^-$ [23]. The autoxidation of hydroquinones also may proceed via a semiquinone and $\cdot O_2^-$ providing an explanation for their ability to break DNA[24]. More recently we have shown that the reduced form of streptonigrin, an antibiotic known to require reduction and oxygen for its action in the cell[25], reacts with oxygen to produce $\cdot O_2^-$ and readily cleaves DNA. Also we have observed the cleavage of DNA by ethidium is strongly dependent on oxygen, as well as light. These results suggest that superoxide may play a role in more reactions than generally appreciated, and suggest possibilities for design in chemotherapeutic agents which, for example, might intercalate DNA as well as give rise to $\cdot O_2^-$ thus highly localizing radical production.

The effects of vitamin C on transforming substance as studied by McCarty[26], in 1944 are remarkably similar to the results presented here. This work seems largely to be unknown and we have only recently ourselves become aware of it. Our data extends and puts on a molecular basis McCarty's results. The relevance to the clinical use of vitamin C is still obscure. Although R17 phage was completely protected by catalase in serum, rhinoviruses responsible for the common cold replicate in the epithelial cells of the upper respiratory tract. It would be important to know the levels of catalase in such tissue. We have found that nasal fluids have sufficient catalase activity to prevent vitamin C cleavage of DNA. Careful studies on intracellular catalase levels and genetic variability are now required. Large doses of vitamin C have been used on patients with terminal cancer[27] with in some cases a remarkable regression of the tumor. One might ask if

these tumors had particularly low levels of catalase. Our results, we hope, will stimulate some further investigation at the molecular level on the very complex roles of vitamin C.

REFERENCES

1 Pauling, L. (1971) Vitamin C and the common cold. W. H. Freeman & Co. San Francisco.
2 Yew, M.-L. S. (1973) Proc. Natl. Acad. Sci. 70, 969.
3 Robinson, A. B., Irving, K. & McCrea, M. (1973) Proc. Natl. Acad. Sci. 70, 2122.
4 Murata, A., Kitagawa, K., Inmaru, H. & Saruno, R. (1972) Agric. Biol. Chem. 36, 2597.
5 Vinograd, J., Lebowitz, J., Radloff, R., Watson, R. & Laipis, P. (1965) Proc. Natl. Acad. Sci USA 53, 1104.
6 Bode, V. C. (1967) J. Mol. Biol. 26, 125.
7 Pouwels, P. H., van Rotterdam, J. and Cohen, J. A. (1969) J. Mol. Biol. 40, 379.
8 Wong, K., Morgan, A. R. and Paranchych, W. (1974) Can. J. Biochem. 52, 950.
9 McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049.
10 Blok, J., Luthjens, L. H. and Roos, A. L. M. (1967) Radiation Res. 30, 468.
11 Fridovich, I. (1972) Accounts of Chemical Res. 5, 321.
12 Greenstock, C. L. Private communication.
13 Raleigh, J. A., Greenstock, C. L. & Kremers, W. (1973) Int. J. Radiat. Biol. 23, 457.
14 Ward, J. F. and Kuo, I. (1973) Int. J. Radiat. Biol. 23, 543.
15 Mayeda, E. A. and Bard, A. J. (1974) J. Am. Chem. Soc. 96, 4023.
16 Allan, R. C., Stjernholm, R. L. and Steele, R. H. (1972) Biochem. Biophys. Res. Commun. 47, 679.
17 Pryor, W. A. "Free Radicals", McGraw-Hill, Inc. p. 136.
18 Curnutte, J. T., Whitten, D. M. and Babior, B. M. (1974) New England J. of Medicine 290, 593.
19 McCord, J. M. (1974) Science 185, 529.
20 Misra, H. P. (1974) J. Biol. Chem. 249, 2151.
21 Lange, C. S. (1974) FEBS Letters 44, 153.
22 Inoue, M., Hayatsu, H. and Tanooka, H. (1972) Chem. Biol. Interactions 5, 85.
23 McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6056.
24 Misra, H. P. and Fridovich, I. (1972) J. Biol. Chem. 247, 188.
25 White, H. L. and White, J. R. (1968) Mol. Pharmacol. 4, 549.
26 McCarty, M. (1945) J. Exp. Med. 81, 501.
27 Cameron, E. and Pauling, L. (1973) Oncology 27, 181.
28 Morgan, A. R. and Pulleyblank, D. E. (1974) Biochem. and Biophys. Res. Comm. 61, 396.
29 Beauchamp, C. O. and Fridovich, I. (1970) J. Biol. Chem. 245, 4661.