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Hydroxyl radical production in body fluids

Roles of metal ions, ascorbate and superoxide

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Hydroxyl radical production, detected by ethylene formation from methional, has been investigated in plasma, lymph and synovial fluid. In the presence of added iron–EDTA, addition of either H_2O_2 or xanthine and xanthine oxidase gave rise to hydroxyl radical formation that in most cases was not superoxide-dependent. The ascorbate already present in the fluid appeared to participate in the reaction. In the absence of added catalyst, the reaction was hardly detectable, the rate being less than 5% of that observed with 1 μM-iron–EDTA added. This implies that the fluids had little if any capacity to catalyse hydroxyl radical production via this mechanism.

It is well recognized that superoxide ($O_2^{-\bullet}$) and H_2O_2 are produced in a wide range of biological reactions involved in the metabolism of O_2 (Fridovich, 1975; Halliwell, 1978*b*). The enzymes superoxide dismutase, glutathione peroxidase and catalase, which break down these activated oxygen metabolites, occur widely, and there is good circumstantial evidence that they protect against the toxic effects of O_2 and are necessary for survival of cells in which oxygen metabolism occurs (McCord *et al.*, 1971; Hassan & Fridovich, 1977; Halliwell, 1978*b*). What is unclear, however, is the actual mechanism of oxygen toxicity (Halliwell, 1978*b*; Fee, 1980). A number of experiments indicate that neither $O_2^{-\bullet}$ nor H_2O_2 is the directly damaging species. Many point to the involvement of a highly reactive species such as the hydroxyl radical ($OH^\bullet$) that could be formed from a combination of the two (Fridovich, 1975; Heikkila *et al.*, 1976; Kellogg & Fridovich, 1977; Cohen, 1977; Weiss *et al.*, 1978; Tauber *et al.*, 1979).

$OH^\bullet$ radicals are produced when H_2O_2 reacts with either $O_2^{-\bullet}$ or ascorbate, provided a suitable metal catalyst is present (McCord & Day, 1978; Winterbourn, 1979; Cohen & Sinet, 1980). It has also been found that $O_2^{-\bullet}$ produced in the presence of ascorbate makes only a minor contribution to $OH^\bullet$ radical production (Winterbourn, 1979). However, it is yet to be established whether either of these mechanisms can operate in biological situations, and whether suitable metal complexes are available to act as catalysts (Cohen & Sinet, 1980; Rigo & Rotilio, 1980).

In the present study $OH^\bullet$ production has been looked for in three different body fluids: plasma, synovial fluid and lymph. It is into this type of extracellular environment that stimulated phagocytes, in an inflammatory situation, would release $O_2^{-\bullet}$ and H_2O_2. Activated oxygen has been implicated in the tissue damage associated with inflammation, and it has been suggested that $OH^\bullet$ could be a major contributor (McCord, 1974; Johnston & Leymeyer, 1976; Halliwell, 1978*a*; Greenwald & Moy, 1980).

We have therefore investigated whether $OH^\bullet$ radicals can be produced from H_2O_2 and either $O_2^{-\bullet}$ or ascorbate in the three fluids. The study was designed to determine (1) whether ascorbate present in the fluid can participate with H_2O_2 in $OH^\bullet$ formation, or whether there is an additional requirement for $O_2^{-\bullet}$, and (2) whether a suitable catalyst is already present in the fluid or needs to be supplied before the reaction can proceed.

$OH^\bullet$ radicals were detected by their reaction with methional to give ethylene (Beauchamp & Fridovich, 1970). Although this reaction is not necessarily specific for $OH^\bullet$ radicals (Pryor & Tang, 1978), other radicals are unlikely to be involved in the present study. The reaction between Fe^{2+} and H_2O_2 is a well-established source of $OH^\bullet$, and this system, along with controls using $OH^\bullet$ scavengers, has previously been used for detecting both ascorbate- and $O_2^{-\bullet}$-dependent $OH^\bullet$ radical production (Cohen & Sinet, 1980; Winterbourn, 1979; Beauchamp & Fridovich, 1970).

Methods

Plasma was obtained from normal human donors. Serum iron, transferrin and caeruloplasmin con-

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centrations were within the normal range. Synovial fluid was obtained by aspiration from the knee joints of rheumatoid-arthritis patients. Cellular constituents were removed by centrifugation. Lymph was collected from mastectomy patients by drainage from the operative site into Redivac containers, generally over the 24h period before analysis.

Differential ascorbate analyses were carried out by the method of Cox & Whichelow (1975). $OH^{\bullet}$ radicals were detected by their reaction with methional to produce ethylene, which was detected by g.l.c. (Winterbourn, 1979). Reactions were carried out in the relevant body fluid diluted from 1.7ml to 2ml by aqueous solutions of methional (to give a concentration of 2mM) and the other reagents specified for each experiment. Reagents and buffers were prepared with deionized distilled water. Rubber-stoppered 12ml tubes were used.

To examine the effects of raising the plasma ascorbate concentration on $OH^{\bullet}$ production, heparinized blood was taken from healthy donors immediately before and 2h after oral administration of 1g of ascorbic acid. $FeSO_4$ was AnalaR grade from BDH, Poole, Dorset, U.K., L-ascorbic acid, EDTA and H_2O_2 were AR grade from Fisons Scientific Apparatus, Loughborough, Leics., U.K.; other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Human oxyhaemoglobin and methaemoglobin preparation methods have been described previously (Winterbourn *et al.*, 1976). Apolactoferrin was purified from human colostrum and reconstituted with Fe^{3+}–nitriloacetate (Querinjean *et al.*, 1971).

Results

Plasma

The reaction between either H_2O_2 and ascorbate or xanthine and xanthine oxidase (which produces a mixture of H_2O_2 and $O_2^{-\bullet}$) in plasma containing methional and catalytic concentrations of Fe^{2+}–EDTA gave rise to ethylene (Fig. 1). The rate of ethylene production was about one-fifth that of an equivalent reaction in buffer, presumably because of the greater competition between plasma constituents and methional for $OH^{\bullet}$ radicals. If Fe^{2+}–EDTA was not added, however, ethylene production from H_2O_2 and either ascorbate or the xanthine oxidase reaction was barely detectable (Fig. 1). As shown in Fig. 2 for ascorbate and H_2O_2, addition of 1–10μM-Fe^{2+}–EDTA gave a progressive increase in the rate of ethylene production,

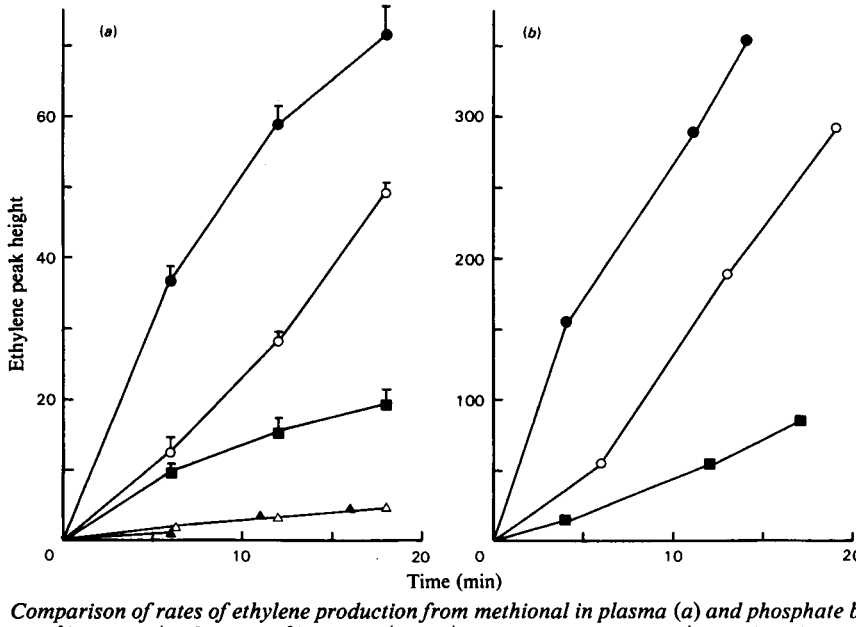

Fig. 1. *Comparison of rates of ethylene production from methional in plasma (a) and phosphate buffer (b)*
Additions: ■, Fe^{2+}–EDTA/H_2O_2; ●, Fe^{2+}–EDTA/H_2O_2/ascorbic acid; ▲, H_2O_2/ascorbic acid; ○, Fe^{2+}–EDTA/xanthine/xanthine oxidase; △, xanthine/xanthine oxidase. Concentrations when present were: 5μM-$FeSO_4$ in 0.1mM-EDTA, 140μM-H_2O_2, 66μM-ascorbic acid, 170μM-xanthine and 0.01 unit of xanthine oxidase/ml. This gives an approximate rate of superoxide generation of 9nmol/ml per min (determined by measuring the rate of cytochrome *c* reduction under conditions where increasing the cytochrome *c*/xanthine oxidase ratio did not increase the rate of reduction). A peak height of 30 corresponds to the production of approx. 10nmol of ethylene/ml.

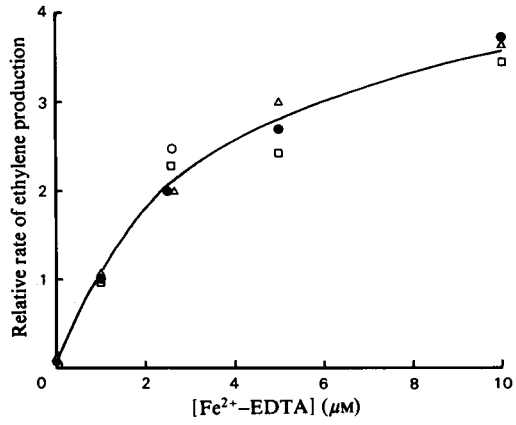

Fig. 2. *Effect of varying Fe^{2+}–EDTA concentration on rate of ethylene production from methional in plasma, lymph and synovial fluid*

Added to each fluid were 67 μM-ascorbic acid, 140 μM-H_2O_2, 10 μM-sodium azide (added to prevent slow H_2O_2 breakdown by endogenous catalase or peroxidase that was found to be present in some fluids) and Fe^{2+}–EDTA as indicated. Gas samples were removed and analysed during the first 20 min of the reaction and the initial rate of increase of peak height was measured. This was linear for approx. 10 min. The rates are expressed relative to that with 1 μM-Fe^{2+}–EDTA added. Each point is the mean of duplicates. Symbols: ●, plasma 1; ○, plasma 2; △, lymph; □, synovial fluid.

levelling out at the higher iron concentrations. In all cases the rate with no addition was only 0.02–0.06 times that in the presence of 1 μM-Fe^{2+}–EDTA. However, even this very low basal rate is of doubtful significance. It was considerably lower than that observed when the equivalent reaction was carried out in buffer; in buffer, omission of 5 μM-Fe^{2+}–EDTA gave an 8-fold decrease in reaction rate, compared with a 60-fold decrease in plasma. In buffer, ethylene is almost invariably produced from methional when H_2O_2 and $O_2^{-\cdot}$ or ascorbate are present, even though they do not react directly to give $OH^\cdot$ radicals. The reaction is inhibited by diethylenetriaminepenta-acetic acid, which forms an iron complex with very low catalytic activity (Halliwell, 1978*a*) and is presumed to be due to contaminant metal ions in either the buffer or reagents. Very little metal ion contamination of the reagents would be required to account for the plasma reaction. A mechanism of ethylene production not dependent on $OH^\cdot$ radicals, such as reported by Weiss *et al.* (1978), could also have been involved. This is suggested by our finding that addition of ascorbate to plasma containing methional gave only a 2-fold increase in the rate of ethylene production, which was only slightly increased by further addition of H_2O_2. In contrast, both H_2O_2 and ascorbate gave a large increase in the presence of Fe^{2+}–EDTA.

Addition of Fe^{2+}–EDTA and H_2O_2 only to plasma gave much slower ethylene production (Fig. 1). Addition of ascorbate (up to 100 μM) gave a concentration-dependent increase in rate. The reaction was H_2O_2-dependent and eliminated by adding catalase, but superoxide dismutase did not significantly inhibit the reaction in fresh plasma, either in the presence or absence of added ascorbate (Table 1). Ethylene production from xanthine and xanthine oxidase in fresh plasma was also almost completely inhibited by catalase and not inhibited by superoxide dismutase (Table 1). The superoxide dismutase was active in plasma, as the same concentration inhibited cytochrome *c* reduction by xanthine and xanthine oxidase in this medium. This contrasts with the $O_2^{-\cdot}$-dependent mechanism of production from Fe^{2+}–EDTA and H_2O_2 in buffer (Table 1) but is the expected result for ascorbate-dependent $OH^\cdot$ production (Winterbourn, 1979).

As shown in Table 1, ethylene production in each instance was inhibited by benzoate and to a lesser extent with ethanol. This pattern of inhibition, which is similar to that observed in buffer (Winterbourn, 1979), is compatible with their acting as $OH^\cdot$ scavengers, and the source of ethylene being the reaction of $OH^\cdot$ with methional. The lower scavenging efficiency in plasma compared with buffer is probably due to the greater number of plasma constituents competing for reaction with $OH^\cdot$.

Some plasma samples had been kept at 4°C for several days before examination. Most of the ascorbate present in these had been oxidized to dehydroascorbate and dioxogulonic acid, and was therefore unable to function as a reducing agent necessary for $OH^\cdot$ radical generation. If no exogenous ascorbate was added, ethylene production was only partially inhibited by superoxide dismutase (Table 1).

These results are compatible with $OH^\cdot$ radical production in plasma supplied with Fe^{2+}–EDTA and H_2O_2, with or without $O_2^{-\cdot}$, occurring via an ascorbate-dependent mechanism. To further test this proposal, a comparison was made between plasmas collected from six individuals before administration of 1 g of ascorbic acid and plasmas collected 2 h later. As shown in Fig. 3, individuals varied in their initial plasma concentrations of total and reduced ascorbate, but in each there was an increase after oral dosage. The rates of ethylene production also varied between individuals, but in each case, and with both Fe^{2+}–EDTA/H_2O_2 and Fe^{2+}–EDTA/xanthine/xanthine oxidase, it was higher in the plasma samples collected after ascorbate administration (Fig. 3). Superoxide dismutase in no instance gave significant inhibition.

Table 1. *Effects of inhibitors on ethylene production from methional in plasma*

Inhibitor	Additions*	Inhibition of ethylene production (%) Fresh plasma†	Stored plasma‡	Buffer
Catalase (20 μg/ml)	Ascorbate	—	97	98
	Xanthine/xanthine oxidase	—	95	97
Superoxide dismutase (15 μg/ml)	H_2O_2	1 ± 13 (7)§	50–60 (3)	60
	H_2O_2/ascorbate	—	2 ± 5 (3)	—
	Xanthine/xanthine oxidase	−6 ± 6 (6)	30–60 (3)	77
	Xanthine/xanthine oxidase/ascorbate	—	0 ± 5 (3)	—
Sodium benzoate (20 mM)	H_2O_2	57	—	—
(33 mM)		80	—	—
(20 mM)	H_2O_2/ascorbate	35	—	56
(33 mM)		75	—	—
Ethanol (15 mM)	H_2O_2	35	—	—
	H_2O_2/ascorbate	11	—	—

* Fe^{2+}–EDTA (5 μM) was added in each case. Other concentrations are the same as in Fig. 1.
† Containing 20–80 μM-reduced ascorbate.
‡ Containing 0–5 μM-reduced ascorbate.
§ Mean ± S.D. Values in parentheses refer to the number of analyses.

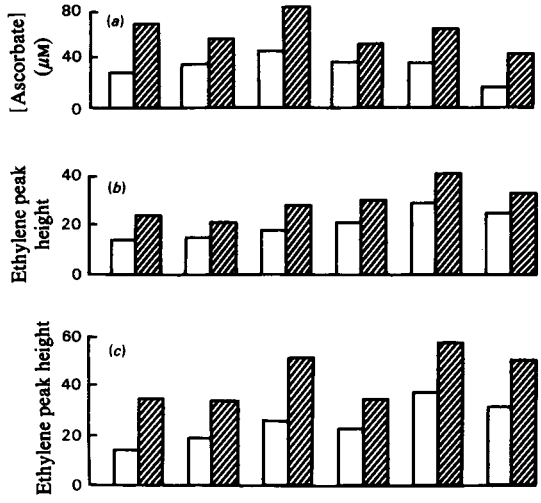

Fig. 3. *Comparison of reduced ascorbate concentrations and rates of ethylene production from methional in plasma before and 2 h after administration of ascorbate to six normal individuals*

(*a*) Plasma reduced ascorbate concentration; (*b*) ethylene produced in 10 min after addition of 2 mM-methional, 10 μM-Fe^{2+}–EDTA, 0.5 mM-xanthine and 0.01 unit of xanthine oxidase/ml; (*c*) ethylene produced in 10 min after adding 2 mM-methional, 10 μM-Fe^{2+}–EDTA and 140 μM-H_2O_2. For calibration of rates of superoxide and ethylene production, see Fig. 1. After administration of oral ascorbate the range of total plasma ascorbate concentrations increased from 76–96 μM to 108–144 μM. □, Before oral administration of 1 g of ascorbic acid; ▨, 2 h after administration of ascorbic acid.

Lymph

The fluid draining into the operative site after mastectomy was collected from five patients. This fluid consists primarily of lymph, but some of the collections contained varying amounts of pigment, presumably products of haemoglobin breakdown in the operative haematoma. The fluids all had biochemical profiles similar to normal plasma, except that the total proteins were about half normal with a higher proportion of albumin. Iron concentrations were in the low normal plasma range. Total ascorbic acid concentrations were from 8 to 20 μM, lower than normally found in plasma, of which 60–100% was oxidized. Much of the oxidation may have occurred in the Redivac containers during the time while the fluid was collecting.

The requirements for ethylene production from methional in lymph were similar to those for plasma. When Fe^{2+}–EDTA was added, both H_2O_2/ascorbate and xanthine/xanthine oxidase gave ethylene production, but in the absence of added catalyst the ethylene produced was only just detectable (Table 2). Further studies of the dependence of ethylene production on Fe^{2+}–EDTA concentration showed that this low basal rate was only about 6% of that with 1 μM-Fe^{2+}–EDTA added, and as with plasma, of doubtful significance (Fig. 2). Even in the fluids containing haemoglobin breakdown products, which might be expected to contain complexed iron, no significant catalytic activity was evident. Most of the fluids contained very low concentrations of reduced ascorbate, and in these, iron-dependent $OH^{\bullet}$ radical production from both H_2O_2 and xanthine/xanthine

Table 2. *Ethylene production from methional in lymph and synovial fluid*
Superoxide dismutase was present at a concentration of 15 μg/ml when indicated.

	Lymph				Synovial fluid	
	(I) No evidence of haematoma		(II) Slight pigmentation			
Reduced ascorbate concn. (μM) ...	8		5		12	
Additions*	Ethylene peak height at 8 min	Inhibition by superoxide dismutase (%)	Ethylene peak height at 8 min	Inhibition by superoxide dismutase (%)	Ethylene peak height at 8 min	Inhibition by superoxide dismutase (%)
Fe^{2+}–EDTA/H_2O_2	20	0 ± 13	14	30	10	0 ± 15
Fe^{2+}–EDTA/H_2O_2/ascorbate	75	0 ± 8	49	0 ± 10	60	—
H_2O_2/ascorbate	2.5	—	—	—	2.5	—
Fe^{2+}–EDTA/xanthine/xanthine oxidase	—	—	13	35	20	—
Xanthine/xanthine oxidase	—	—	0.5	—	—	—

* Concentrations: 10 μM-$FeSO_4$, 1 mM-EDTA, 140 μM-H_2O_2, 67 μM-ascorbate, 0.17 mM-xanthine, and 0.01 unit (for lymph) or 0.02 unit (for synovial fluid) of xanthine oxidase/ml. All solutions contained 2 mM-methional.

oxidase was partially inhibited by superoxide dismutase (e.g. Table 2, II). The one sample obtained directly by aspiration rather than Redivac collection (Table 2, I) contained some reduced ascorbate, and in this case there was no inhibition by superoxide dismutase.

Synovial fluid

Synovial fluid was obtained from four rheumatoid-arthritis patients. The samples varied in viscosity, indicating a variation in inflammatory status of the patients, but all gave similar results. Total ascorbate concentrations of 52–58 μM, which are near the middle of the normal plasma range, were found, but greater proportions were oxidized (64–78%) than generally found in plasma.

Addition of H_2O_2 and ascorbate to each synovial fluid gave ethylene production from methional, but only if Fe^{2+}–EDTA was also added. As with plasma and lymph, the catalytic activity of synovial fluid was less than 5% that of 1 μMFe^{2+}–EDTA (Fig. 2). Addition of Fe^{2+}–EDTA and H_2O_2 to synovial fluid also resulted in ethylene production, but much more slowly than when ascorbate was also added. The results of one experiment are shown in Table 2. No inhibition by superoxide dismutase was apparent. Ethylene was also produced from Fe^{2+}–EDTA, xanthine and xanthine oxidase. Inhibition by superoxide dismutase was studied in one synovial fluid that had been kept at 4°C and contained only 4 μM-reduced ascorbate. In this fluid, with 20 nmol of $O_2^{-\cdot}$ generated/ml per min, superoxide dismutase inhibited the reaction by 50%. Benzoate and ethanol both inhibited ethylene production.

Haemoglobin and lactoferrin

Haemoglobin, which could be found in body fluids as a result of erythrocyte haemolysis, and lactoferrin, which is present in various secretions as well as the granules of neutrophils, were investigated as potential catalysts of $OH^{\cdot}$ production. Addition of H_2O_2 to either oxyhaemoglobin or methaemoglobin resulted in the production of ethylene from methional. The rate was faster with methaemoglobin than with oxyhaemoglobin. However, the reaction was inhibited by cyanide, but unaffected by superoxide dismutase, benzoate or ethanol. It was most likely due, therefore, to peroxidation of methional by methaemoglobin, and in this case ethylene production is not indicative of $OH^{\cdot}$ formation.

No catalysis of $OH^{\cdot}$ formation (ethylene production from methional) from either xanthine and xanthine oxidase or H_2O_2 and ascorbate was observed with up to 5 μM-lactoferrin (10 μM with respect to iron), either at pH 7.4 or pH 5 (approximately the pH inside neutrophil phagocytic vacuoles).

Discussion

Simultaneous production of $O_2^{-\bullet}$ and H_2O_2 has frequently been found to lead to the production of $OH^\bullet$ radicals (Fridovich, 1975; Cohen, 1977; Halliwell, 1978*b*). This does not arise from the direct reaction between H_2O_2 and $O_2^{-\bullet}$, which is much too slow (Halliwell, 1976; Rigo *et al.*, 1977), and a transition metal ion catalyst must be added or present as a contaminant (McCord & Day, 1978; Cohen & Sinet, 1980; Rigo & Rotilio, 1980; Fee, 1980). Iron–EDTA and iron–ADP are good catalysts, and under these conditions the reaction is the well-known Fenton reaction:

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^\bullet$$

The $O_2^{-\bullet}$ serves as a reducing agent to regenerate the Fe(II) ions:

$$Fe(III) + O_2^{-\bullet} \rightarrow Fe(II) + O_2$$

Ascorbate can also act in the capacity of a reducing agent (Udenfriend *et al.*, 1954; Winterbourn, 1979), and circumvent the requirement for $O_2^{-\bullet}$, but as with $O_2^{-\bullet}$ the direct reaction between ascorbate and H_2O_2 is not a significant source of $OH^\bullet$ radicals.

Non-biological media frequently have sufficient metal ion contaminants present for these reactions to proceed at a measurable rate, but it has not been established whether suitable catalysts are available in biological fluids. In the present study, three major human body fluids, plasma, lymph and rheumatoid synovial fluid, were examined. Hydroxyl radicals were detected by their reaction with methional to give ethylene and were found to be produced in each fluid when H_2O_2 and $O_2^{-\bullet}$ or ascorbate, along with an Fe^{2+}–EDTA catalyst, were present. In the absence of added catalyst, ethylene production in all the fluids examined was low enough to be accounted for by metal ion contaminants in the reagents or $OH^\bullet$-independent mechanisms. One possible interpretation of this result is that $OH^\bullet$ radicals could have been produced at low concentrations, but undetected because of scavenging by fluid constituents such as caeruloplasmin. For this to be the case, however, there should have been a threshold iron concentration below which no $OH^\bullet$ production was detected. There was no indication of this. Hence although scavengers in the fluids competed with methional to decrease the ethylene yield, they apparently did not prevent this reaction. Since the relationship between ethylene production and Fe^{2+}–EDTA concentration below about 2μM was almost linear, it can be concluded that even if the basal rate of ethylene production in the fluids did represent metal catalysis of $OH^\bullet$ production, any endogenous catalysts present were less active than 0.05μM-Fe^{2+}–EDTA. This suggests that transferrin (present at approx. 20μM in iron in plasma) is either not a catalyst of the reaction, or only a very poor one, in spite of a previous report to the contrary (McCord & Day, 1978). Haem breakdown products, arising from post-operative haematomas, and two other iron-containing proteins, haemoglobin and lactoferrin from human milk, also showed no detectable catalytic activity.

The lack of significant endogenous metal catalysis of $OH^\bullet$ production in inflammatory synovial fluid is interesting in relation to studies of hyaluronic acid depolymerization by H_2O_2 and $O_2^{-\bullet}$. This is thought to involve $OH^\bullet$ radicals (McCord, 1974) and with isolated hyaluronic acid, is dependent on metal ion catalysis (Halliwell, 1978*a*; Greenwald & Moy, 1980). The present findings suggest that the depolymerization observed in diluted synovial fluid (McCord, 1974) may have been catalysed by metal complexes added with the reagents, or a mechanism not involving metal-ion-catalysed production of free $OH^\bullet$ radicals may be involved.

Although no evidence that normal body fluid constituents can catalyse the Fenton reaction has been found, there may be conditions that allow the reaction to proceed. There is evidence that low-molecular-weight iron complexes occur as metabolic intermediates, although little is known of their nature (Jacobs, 1977). Intracellular complexes such as iron–ADP, which could give rise to $OH^\bullet$ radicals, are therefore possible. These complexes may also be increased or more widespread in iron overload (Hershko *et al.*, 1978). In such circumstances, it is of interest whether there is also a requirement for $O_2^{-\bullet}$, and hence whether the prevention of such a reaction could be an important function of superoxide dismutase. Although this implication is frequently made, recent assessments by Fee (1980) and Rigo & Rotilio (1980) suggest that it is not supported by the information available. A similar conclusion must be made from the present findings that ascorbate already present in the fluid can act as the necessary reducing agent, and that the only additional requirement for $OH^\bullet$ production is a source of H_2O_2. In plasma, lymph and synovial fluid, provided the fluid was freshly collected, $OH^\bullet$ radical formation was not inhibited by superoxide dismutase, even when a source of O_2^- was provided, and reduced ascorbate concentrations were high enough to account for the observed rates. Further evidence that ascorbate is a major contributor to iron-catalysed $OH^\bullet$ radical production was obtained in plasma taken from individuals before and after oral administration of ascorbic acid. This dose raised both the total and reduced ascorbate concentrations, but with only 2h between blood samples, other plasma constituents were probably only minimally changed. In all cases the increase in ascorbate concentration was associated with an increase in $OH^\bullet$ radical production.

Only in fluids containing little or no reduced ascorbate, or when the rate of $O_2^{-\bullet}$ generation was

very high, was any $O_2^{-\bullet}$-dependence found. Most of the low reduced ascorbate concentrations encountered in the present studies could have been due to oxidation occurring on storage, although the high proportions of oxidized ascorbate measured in the synovial fluid and aspirated lymph samples may have arisen *in vivo*. Ascorbate concentrations could also be low in rheumatoid arthritis, and in these circumstances $O_2^{-\bullet}$-dependent $OH^\bullet$ production could be more important particularly at high rates of $O_2^{-\bullet}$ generation. Biologically $O_2^{-\bullet}$ production is likely to be high with localized concentrations of activated neutrophils. However, the ubiquitous occurrence of ascorbate, both intra- and extracellularly, leads to the conclusion that even then an ascorbate-dependent mechanism would be likely to predominate. After the ascorbate had been destroyed by the oxidative process, the reaction could become more $O_2^{-\bullet}$-dependent, but nevertheless, the overall effect of superoxide dismutase would be to decrease $OH^\bullet$ production but not prevent it. An essential role for superoxide dismutase in preventing this reaction would therefore seem unlikely.

Although the present findings do not support the Fenton reaction as an explanation for $O_2^{-\bullet}$ toxicity in biological systems, they do not preclude other mechanisms of $O_2^{-\bullet}$-dependent $OH^\bullet$ radical production. These may be of major significance as, for example, suggested by recent reports of $OH^\bullet$ radical production by activated neutrophils being inhibited by superoxide dismutase but not by catalase (Tauber *et al.*, 1979; Green *et al.*, 1979).

Unless more positive evidence for the existence of suitable metal catalysts can be obtained, $OH^\bullet$ radical production via the Fenton reaction cannot be assumed to occur in biological fluids, or be used as an explanation for tissue-damaging reactions. The significance of $O_2^{-\bullet}$ in this process must also be in doubt. The results of the present study support ascorbate as a more likely biological $OH^\bullet$ radical producer. Phagocytic leucocytes contain very high concentrations of ascorbate, and speculation that it might be involved in the microbicidal mechanism of these cells (Miller, 1969; McCall *et al.*, 1971) warrants further consideration.

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