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Increase in Microvascular Permeability Induced by Enzymatically Generated Free Radicals

II. Role of Superoxide Anion Radical, Hydrogen Peroxide, and Hydroxyl Radical[1]

R. F. DEL MAESTRO,*[,2,3] J. BJÖRK,† AND K.-E. ARFORS‡

‡*Department of Experimental Medicine, Pharmacia AB, S-751 04 Uppsala, Sweden; *Brain Research Laboratory, Department of Clinical Neurological Sciences, Victoria Hospital, London, Ontario N6A 4G5, Canada; and †Burn Centre, University Hospital, S-750 14 Uppsala, Sweden*

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The roles played by superoxide anion radical (O_2^-), hydrogen peroxide, and hydroxyl radical (OH·) in the macromolecular permeability increase seen after addition of xanthine oxidase to the hamster cheek pouch has been studied. Fluorescein-labeled Dextran, $\bar{M}_w$ 150,000 (FITC–Dextran 150) was used to assess microvascular permeability. Application of xanthine oxidase was associated with a marked increase in the observed FITC–Dextran 150 leakage sites per square centimeter. This macromolecular extravasation was significantly reduced by the placement in the reservoir fluid of 50 μg/ml superoxide dismutase, an O_2^- scavenger, 50 μg/ml catalase, and the OH· scavenger dimethyl sulfoxide, and especially by L-methionine which can react with both OH· and quench singlet O_2. These findings suggest that O_2^- and H_2O_2 generated during endogenous substrate–xanthine oxidase reactions result in further generation of OH· which causes the increased macromolecular extravasation possibly mediated through the interactions of OH· with plasmalemmal lipids. A concept of the roles of the individual radical species and their products is presented in the hope that it may aid in the understanding and treatment of inflammatory conditions.

INTRODUCTION

The application of xanthine oxidase alone or in combination with the exogenous substrate, hypoxanthine, to the cheek pouch of the hamster was associated with a range of microvascular alterations which are also observed during the inflammatory process (Del Maestro *et al.*, 1981). Macromolecular extravasation, arteriolar vasoconstriction, and increased leukocyte rolling were seen. The hypothesis was suggested that some of these changes were related to the free radical flux associated with the aerobic oxidation of exogenous and/or endogenous substrates by the enzyme. Since the activation of polymorphonuclear leukocytes, macrophages, and monocytes (Babior, 1978) results in release with subsequent generation into the extracellular space of the same radical species it was further

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[2] Recipient of Canadian Medical Research Council Fellowship.
[3] To whom reprint requests should be sent.

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suggested that these radicals play a role in the permeability alterations seen during inflammation.

The purpose of this study was to elucidate the role of superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) in the macromolecular permeability changes observed after application of active xanthine oxidase to the cheek pouch. To study the role of these individual molecular species we have used the enzymatic scavenger superoxide dismutase (SOD) which removes O_2^- (McCord and Fridovich, 1969), catalase (CAT) which reduces H_2O_2 to H_2O, and molecules which scavenge OH·.

MATERIALS AND METHODS

Enzymes

Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2, Grade I, 0.66 unit/mg), catalase (hydrogen-peroxide:hydrogen-peroxide reductase, EC 1.11.1.6), and superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) were obtained from Sigma Chemical Company, St. Louis, Missouri. Xanthine oxidase was denatured by heating at 100° for 20 min.

Enzyme Assays

SOD was assayed as described by Crapo *et al.* (1978) and had a specific activity of 1700 units/mg protein. CAT had an activity of 31,700 units/mg protein as measured by the method of Beers and Sizer (1952).

Chemicals

Cytochrome c^{3+} (Type VI) and hypoxanthine were purchased from Sigma Chemicals while L-methionine, H_2O_2, and buffer salts were obtained from E. Merck, Darmstadt, West Germany. Dimethyl sulfoxide (DMSO) was a gift from Dr. Stanley Jacob.

Solutions

The bicarbonate-buffered superfusion fluid contained (millimolar): NaCl, 131.9; KCl, 4.7; $CaCl_2$, 2.0; $MgSO_4$, 1.2; and $NaHCO_3$, 18. The solutions used for both *in vitro* and *in vivo* experiments were the bicarbonate-buffered solution described unless otherwise stated. All bicarbonate solutions were maintained at pH 7.35, P_{O_2} between 2–4 pK_a by bubbling with 5% CO_2 and 95% N_2 and at 37°.

Preparation of Animals

Male golden hamsters (*Mesocricetus auratus*) weighing 70–120 g obtained from Stockholms Biologiska Laboratorium were used. They were anesthetized with sodium pentobarbital (Nembutal, Abbott, Chicago, Ill., 60 mg/100 g body wt) intraperitoneally. The animals were prepared as described elsewhere (Del Maestro *et al.*, 1981) and transferred to the stage of a Leitz Ortholux microscope. FITC–Dextran 150 (fluorescein-labeled Dextran, $\bar{M}_w$ = 150,000) obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, was injected intravenously (25 mg/100 g) as a 5% solution in 0.9% NaCl.

Permeability Studies

Macromolecular extravasation was assessed in a semiquantitative manner by counting the macromolecular leakage sites (Del Maestro *et al.*, 1981) after intravenous administration of FITC–Dextran 150 both in the 60-min equilibration period and following application of test solution(s) to the cheek pouch. The sequence of application of the test solution(s) was randomized and a standardized technique was used for all animals.

(1) Xanthine oxidase. Superfusion was discontinued and a 1.0-ml solution of active xanthine oxidase was added to the reservoir solution to obtain a concentration of 0.05 unit/ml enzyme.

(2) Text solutions. The superfusions were stopped and the reservoir solution surrounding the cheek pouch carefully replaced with freshly prepared bicarbonate buffer solutions containing either (i) SOD 10 μg/ml, (ii) SOD 50 μg/ml, (iii) CAT 50 μg/ml, (iv) DMSO 10 m*M*, or (v) L-methionine 10 m*M* to which was immediately added 1.0 ml of active xanthine oxidase solution to give a final concentration of 0.05 unit/ml enzyme in the reservoir.

In each experiment the xanthine oxidase solution was allowed to remain in contact with the cheek pouch for 1 min and then removed with the recommencing of the superfusion. Leakage sites were counted at 5, 10, 15 min and thereafter at 10-min intervals for a total experimental time of 85 min.

Assay of Superoxide Generation

The *in vitro* reduction of cytochrome c^{3+} (Cyt c^{3+}) by O_2^- was assessed as described elsewhere (Del Maestro *et al.*, 1981). The system was composed of 0.96 m*M* hypoxanthine, 50 μ*M* Cyt c^{3+} in bicarbonate buffer to which xanthine oxidase (0.05 unit/ml) was added in a final volume of 2.5 ml.

Superoxide anion radical generation was also assessed in the reservoir surrounding the cheek pouch. This was carried out in two series of experiments.

Series I. The superfusion was discontinued and the reservoir fluid replaced with a series of solutions contained in 10 ml of bicarbonate buffer:

(i) 50 μ*M* Cyt c^{3+};

(ii) 50 μ*M* Cyt c^{3+} and then 1.0 ml of denatured enzyme was applied to give a 0.05 unit/ml reservoir concentration;

(iii) 50 μ*M* Cyt c^{3+} and 0.96 m*M* hypoxanthine;

(iv) 50 μ*M* Cyt c^{3+} and 0.96 m*M* hypoxanthine to which the 1.0-ml solution of active xanthine oxidase was added to give a 0.05 unit/ml reservoir concentration;

(v) 50 μ*M* Cyt c^{3+}.

Series II. The superfusion was discontinued and replaced with solutions containing:

(i) 50 μ*M* Cyt c^{3+};

(ii) 50 μ*M* Cyt c^{3+} to which active enzyme was added (0.05 unit/ml);

(iii) 50 μ*M* Cyt c^{3+}.

Each solution was left in contact with the pouch for 1 min; superfusion was then restarted and continued for 10 min before application of the next solution in the series. After obtaining two 0.2-ml control samples, further sampling was carried out from the reservoir at 5, 10, 15, 30, and 60 sec after application of the test solution(s). All samples were immediately frozen in a bath containing

dry ice and acetone, stored at −20°, and later absorbance was compared to controls at 550 nm.

Assay of Uric Acid Production

Uric acid produced from hypoxanthine by xanthine oxidase was determined in the *in vitro* O_2^--generating system by assaying the absorbance increase at 290 nm ($\Delta\varepsilon_{290}$ = 14,000 M^{-1} cm^{-1}).

Reaction of Compounds with H_2O_2

The reaction of substances with H_2O_2 was measured by a fall in absorbance at 240 nm as H_2O_2 is destroyed (Beers and Sizer, 1952).

Statistical Methods

Measurements quoted are mean values ± SEM. For the *in vitro* data statistical significance was calculated using the Student *t* test and a *P* value <0.05 was considered significant. Statistical significance was calculated using the rank–sum test for the cheek pouch data with an α value <0.025 considered significant (Dixon and Massey, 1957).

RESULTS

The use of fluorescent microscopy and 35× magnification allowed continuous observation of the hamster cheek pouch microvasculature. Within 20 sec of intravenous injection FITC–Dextran 150 appeared in all cheek pouch preparations and was observed in all microvascular vessels within 35 sec.

Influence of Xanthine Oxidase on FITC–Dextran Extravasation

The application of active xanthine oxidase to the hamster cheek pouch was associated with an increase in the number of leakage sites per square centimeter which reached a maximum 10 min (Fig. 1) after addition and then decreased over the remaining 75 min. The majority of the leakage sites were in the region of

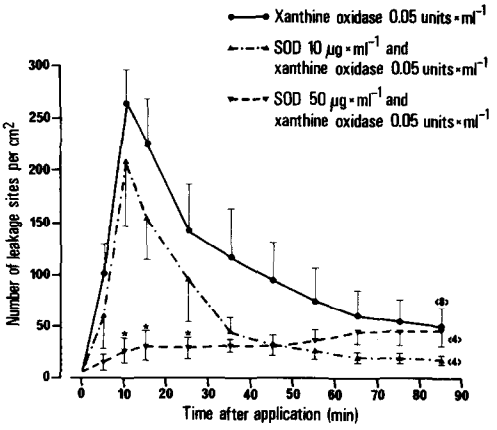

FIG. 1. Mean number ± SE of FITC–Dextran 150 leakage sites/cm^2 after addition of 10 and 50 μg/ml SOD to the reservoir before application of xanthine oxidase compared to controls in which no SOD was added. Number of brackets is the number of animals in each group. Values for the SOD groups are compared to the xanthine oxidase-alone group at each time peroid using the rank–sum test. Significant difference (*) is indicated when α < 0.025.

the postcapillary venules with some extravasation seen from larger veins (>20 μm) as described previously (Del Maestro *et al.*, 1981). FITC–Dextran 150 extravasation was not observed from arteries, arterioles, or capillaries and no petechial hemorrhages were observed.

Effect of SOD on Xanthine Oxidase-Induced Permeability Increase

The incorporation of 10 μg/ml SOD in the cheek pouch reservoir fluid was related to a small decrease in mean leakage sites per square centimeter compared to the addition of xanthine oxidase alone (Fig. 1). These decreases were not statistically significant. The addition of 50 μg/ml SOD to the cheek pouch reservoir prior to application of xanthine oxidase was associated with statistically significant (Fig. 1) decreases in the mean number of leakage sites per square centimeter at three time intervals studied. The presence of 50 μg/ml SOD in the reservoir fluid resulted in a 91 ± 5% decrease in the mean number of leakage sites per square centimeter 10 min following application of active enzyme (Fig. 2).

Effect of Catalase

The addition of 50 μg/ml catalase to the reservoir prior to addition of xanthine oxidase was associated with a significant decrease in leakage sites per square centimeter (Fig. 3). An 84 ± 8% decrease in leakage sites was observed at 10 min (Fig. 2).

Effect of DMSO and L-Methionine

The addition of either 10 m*M* DMSO or 10 m*M* L-methionine to the reservoir fluid both significantly lowered the expected number of leakage sites (Fig. 4) resulting in a 74 ± 10 and a 92 ± 4% decrease at 10 min, respectively (Fig. 2). L-methionine was the most effective of the agents tested in preventing FITC–Dextran 150 macromolecular extravasation.

Absorbance Changes in Cyt c^{3+} after Addition of Test Solution(s) to the Cheek Pouch Reservoir

Changes in Cyt c^{3+} absorbance at 550 nm after application of the test solution(s) in Series I and II can be seen in Tables 1 and 2, respectively. Reduction of Cyt

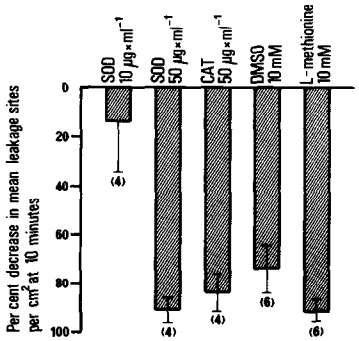

FIG. 2. Percent decrease in the mean leakage sites/cm² (±SE) at 10 min following application of 0.05 unit/ml of xanthine oxidase in the presence of the substances tested as compared to the mean leakage site/cm² in their absence. Number in brackets represents number of animals.

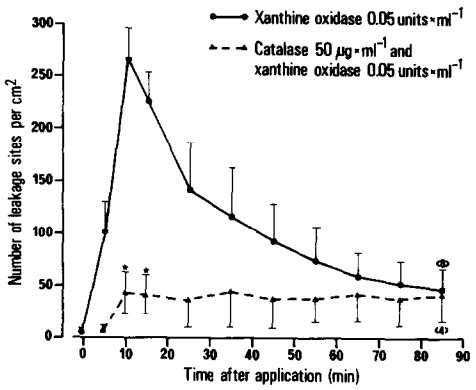

FIG. 3. Values are means ± SE of leakage sites/cm² with and without addition of 50 μg/ml catalase to the reservoir before addition of active enzyme. Number in brackets represents number of animals. Values for the catalase group are compared to the xanthine oxidase-alone group at each time interval studied using the rank–sum test and significant difference (*) is indicated when $\alpha < 0.025$

c^{3+} by O_2^- would result in an increase in 550 nm absorbance while oxidation of any Cyt c^{2+} present in the solution results in a decrease from control values. In both Series I and II the initial replacement of the reservoir fluid with one containing Cyt c^{3+} in bicarbonate buffer under the experimental conditions for 1 min resulted in no absorbancy change from control values. No changes were also noted with the presence of denatured xanthine oxidase (0.05 unit/ml) or 0.96 m*M* hypoxanthine. Active xanthine oxidase (0.05 unit/ml) addition to the reservoir fluid surrounding the cheek pouch containing 0.96 m*M* hypoxanthine and 50 μ*M* Cyt c^{3+} resulted in large mean increases in absorbance at 550 nm which increased over the first 15 sec and then decreased (Table 1). In two of four hamsters in which xanthine oxidase was added to the reservoir fluid without

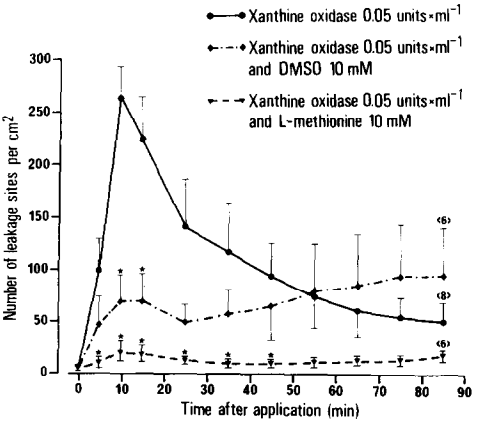

FIG. 4. Values are means ± SE of leakage sites/cm² after addition of 10 m*M* DMSO and 10 m*M* L-methionine to the reservoir before addition of enzyme. Number in brackets represents number of animals. Leakage-site values for these groups are compared with the xanthine oxidase-alone group for each time period using the rank–sum test and significant difference (*) is indicated when $\alpha < 0.025$.

TABLE 1

ABSORBANCE CHANGE OF CYT c^{3+} AT 550 nm AFTER TOPICAL APPLICATION OF TEST SOLUTION(S) IN SERIES I

Time after application (sec)	Cyt c^{3+}	Cyt c^{3+}, denatured xanthine oxidase (0.05 unit/ml)	Cyt c^{3+}, hypoxanthine (0.96 m*M*)	Cyt c^{3+}, hypoxanthine (0.96 m*M*), xanthine oxidase (0.05 unit/ml)	cyt c^{3+}
5	0	0	0	0.32 ± 0.06	−0.01 ± 0.01
10	0	0	0	0.39 ± 0.04	−0.03 ± 0.02
15	0	0	0	0.44 ± 0.06	−0.02 ± 0.01
30	0	0	0	0.24 ± 0.06	−0.02 ± 0.01
60	0	0	0	0.21 ± 0.07	−0.03 ± 0.01

Note. Values are mean Δ absorbance of 50 μ*M* Cyt c^{3+} at 550 nm ± SEM compared to controls as described under Materials and Methods; four observations were made at each time interval. Solutions were left in contact with the cheek pouch for 1 min with 10 min elapsing between applications.

TABLE 2
ABSORBANCE CHANGE OF CYT c^{3+} AT 550 nm AFTER TOPICAL APPLICATION OF TEST SOLUTION(S) IN SERIES II

Time after application (sec)	Cyt c^{3+}	Cyt c^{3+}, xanthine oxidase (0.05 unit/ml)	Cyt c^{3+}
5	0	0.01 ± 0.01	0
10	0	0.02 ± 0.01	0
15	0	0.02 ± 0.01	0
30	0	0.03 ± 0.02	0
60	0	0.01 ± 0.01	0

Note. Values are mean Δ absorbance of 50 μ*M* Cyt c^{3+} at 550 nm ± SEM compared to controls as described under Materials and Methods; four observations were made at each time interval. Solutions were left in contact with the cheek pouch for 1 min with 10 min elapsing between applications.

exogenous substrate a small increase in absorbance at 550 nm was noted and the mean values for the time intervals assessed are given in Table 2. In three of the four experiments in which Cyt c^{3+} was added to the reservoir 10 min after the addition of active xanthine oxidase to the hypoxanthine solution a small decrease was noted in absorbance (Table 1). No changes were noted 10 min after addition of active xanthine oxidase alone to the reservoir of the hamster cheek pouch (Table 2).

In Vitro Uric Acid and O_2^- Formation

The *in vitro* uric acid formation from hypoxanthine by xanthine oxidase was not significantly decreased by any of the compounds employed (Table 3). The substances used therefore did not inhibit *in vitro* xanthine oxidase activity. L-Methionine significantly increased the rate of uric acid formation in these experiments (Table 3).

During these experiments we also attempted to monitor the production of xanthine to investigate the accumulation of this intermediate during the reaction.

TABLE 3
RATE OF *IN VITRO* O_2^- AND URIC ACID FORMATION

	O_2^- formation (nmole/min/ml)	Uric acid formation (nmole/min/ml)
Control	12.0 ± 0.5	12.9 ± 0.7
SOD 10 μg/ml	4.9 ± 0.3*	12.8 ± 0.2
SOD 50 μg/ml	3.3 ± 0.1*	13.1 ± 0.2
CAT 50 μg/ml	10.5 ± 0.5	12.7 ± 0.4
DMSO 10 m*M*	10.4 ± 0.7	13.7 ± 0.3
L-Methionine 10 m*M*	11.0 ± 0.3	16.3 ± 0.3*

Note. The assay mixtures contained 0.96 m*M* hypoxanthine, 50 μ*M* Cytochrome c^{3+} in bicarbonate buffer (pH 7.4) to which xanthine oxidase (0.05 unit/ml) was added to initiate the reaction. Test compounds were added to this mixture prior to xanthine oxidase. Values are means ± SEM for six experiments in each case and O_2^- and uric acid generation rates calculated as described under Materials and Methods. Statistically significant differences from control values were calculated using the Student *t* test and are indicated (*) when $P < 0.05$.

Using spectrophotometric means we were unable to detect its presence during the reaction. This suggested that under our *in vitro* reaction conditions hypoxanthine was oxidized to uric acid without the accumulation of the intermediate xanthine. Table 3 also shows the results of the presence of these compounds on O_2^- formation. *In vitro* O_2^- generation was significantly reduced only in the presence of SOD (10 and 50 μg/ml).

In Vitro Reactions with H_2O_2

The addition of 50 μg/ml SOD, 10 m*M* DMSO, or 10 m*M* L-methionine to the H_2O_2 solution resulted in no change in absorbance at 240 nm. These substances under the *in vitro* test conditions and in the concentrations used did not react with H_2O_2. The addition of 50 μg/ml CAT to the H_2O_2 solution resulted in an immediate decrease in 240-nm absorbance with the visible generation of gas in the cuvette.

DISCUSSION

These experiments indicate that the application of active xanthine oxidase to the hamster cheek pouch results in an increase in FITC–Dextran 150 leakage sites per square centimeter. The mean number of leakage sites observed was significantly decreased at some time intervals by the incorporation of SOD 50 μg/ml, CAT 50 μg/ml, 10 m*M* DMSO, and 10 m*M* L-methionine in the reservoir fluid prior to application of the xanthine oxidase.

Considerations of Methods

The cheek pouch of the hamster has been used extensively for studies related to the microcirculation since it was described by Fulton *et al.* (1946). Except for its lack of lymphatics (Handler and Shepro, 1968) the cheek pouch possesses the array of vascular and nervous elements present in other connective tissues. The cheek pouch, if carefully prepared and continually superfused, can be observed by *in vivo* microscopy for more than 3 hr without significant deterioration (Duling, 1973; Svensjö *et al.*, 1978). The intravenous injection of FITC–Dextran 150 (Svensjö *et al.*, 1978) as a macromolecular permeability marker and the use of fluorescent microscopy allows a semiquantitative assessment of macromolecular extravasation by counting the number of leakage sites per square centimeter and also allows accurate delineation of the site of FITC–Dextran 150 extravasation. More general observations can also be made on vessel diameters and leukocyte behavior. The experimental manipulations of exchanging the reservoir fluid surrounding the cheek pouch and discontinuing the superfusion for 1 min followed by recommencing superfusion did not result in significant increases in the number of leakage sites observed (Del Maestro *et al.*, 1981). This model tissue appeared adequately suited for the experimental purpose of studying the influence of extracellular generation of free radicals on the microcirculation.

Substrate–Xanthine Oxidase Free Radical Generation

The substrate–xanthine oxidase model for the generation of free radicals has been intensively studied (Fridovich, 1970) and employed to generate free radicals both *in vitro* (Kellogg and Fridovich, 1975, 1977) and during *in vivo* studies

(Ohmori *et al.*, 1978). Rosen and Klebanoff (1979) have employed an acetylaldehyde–xanthine oxidase system as a model for polymorphonuclear leukocytes to study the bactericidal effects of the free radicals generated. The mechanisms of free radical generation during these substrate–enzyme interactions is not completely understood. Fridovich (1970) has outlined both a univalent pathway resulting in O_2 reduction to O_2^- (reaction 1) and a divalent pathway resulting in the two electron reduction of O_2 to H_2O_2 (reaction (2)).

$$\text{Enz–H}_2 + 2O_2 \rightarrow \text{Enz} + 2O_2^- + 2H^+, \quad (1)$$

$$\text{Enz–H}_2 + O_2 \rightarrow \text{Enz} + H_2O_2. \quad (2)$$

A prerequisite for the assessment of free radical influences on the microvasculature was that free radicals were generated and then able to interact with the cheek pouch under the experimental conditions employed. This was highlighted during initial investigations using Tris buffer for *in vitro* and *in vivo* experiments. Tris buffer may interact with the radicals produced resulting in significant scavenging during *in vitro* experiments (Fridovich and Handler, 1962) and a decreased ability of the hypoxanthine–xanthine oxidase system to induce macromolecular extravasation. *In vitro* experiments using bicarbonate buffer superfusion solution and the hypoxanthine–xanthine oxidase system resulted in O_2^- generation as measured by Cyt c^{3+} reduction (Table 3). These results were consistent with the finding that Cyt c^{3+} reduction also occurred when active xanthine oxidase was added to the reservoir fluid containing hypoxanthine (Table 1). Activity of the enzyme appeared essential since no Cyt c^{3+} reduction occurred in the presence of denatured enzyme either on the surface of the pouch (Table 1) or during *in vitro* experiments (Del Maestro *et al.*, 1981). *In vitro* studies using cheek pouch homogenates in the absence of exogenous substrates resulted in O_2^- generation (Del Maestro *et al.*, 1981). A small increase in Cyt c^{3+} reduction in the reservoir fluid was seen during two of four experiments in which xanthine oxidase was added (Table 2). Many other substances present in the cheek pouch may react with the generated O_2^- and only a small percentage of the O_2^- may be available to reduce Cyt c^{3+} in the reservoir fluid. These endogenous substrate–enzyme interactions would also be expected to generate H_2O_2 (reaction (2)) and spontaneous dismutation of O_2^- would result in further H_2O_2 formation via reaction 3 (McCord and Fridovich, 1969).

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2. \quad (3)$$

The application of xanthine oxidase to the reservoir fluid containing hypoxanthine and Cyt c^{3+} resulted in the initial reduction of Cyt c^{3+} to Cyt c^{2+} by O_2^- followed by reoxidation of some of this Cyt c^{2+} (Table 1). This suggests that an oxidizing agent is formed which can oxidize the Cyt c^{2+} produced. Substrate–xanthine oxidase systems have been shown to generate OH· (Fong *et al.*, 1976) which could readily oxidize Cyt c^{2+}. A great deal of controversy has centered on the mechanism of the further generation of OH· from O_2^- and H_2O_2 (Fee and Valentine, 1977; Cohen, 1977). The reduction of metal complexes by O_2^- (reaction (4)) appears to be an essential component of this reaction sequence and the further generation of OH· (reaction (5)) may occur via a Fenton-type reaction (McCord and Day, 1978).

$$Me^{n+} \text{ chelate} + O_2^- \longrightarrow Me^{(n-1)+} \text{ chelate} + O_2, \quad (4)$$

$$Me^{(n-1)+} \text{ chelate} + H_2O_2 \rightarrow Me^{n+} \text{ chelate} + OH\cdot + OH^-. \quad (5)$$

A decrease in absorbance at 550 nm was found in three of four experiments 10 min following application of xanthine oxidase to the cheek pouch reservoir fluid containing hypoxanthine and Cyt c^{3+} (Table 1). Some substance(s) capable of oxidizing the Cyt c^{2+} found in the Cyt c^{3+} solution must be present at this time since this oxidation was not found in other experiments in which the Cyt c^{3+} solution alone was added to the cheek pouch reservoir. Since radical species are very short lived (Pryor, 1976) it seems unlikely that any radical species generated during the reaction could still be present 10 min following the reaction. It would appear that some oxidizing product(s) of the reaction may be available at this time to oxidize Cyt c^{2+}.

It has been suggested that singlet oxygen (O_2 ($^1\Delta g$)), an activated and more reactive O_2 species, is also generated during xanthine oxidase reactions (Kellogg and Fridovich, 1975, 1977). At present both the generation and the possible role of O_2 ($^1\Delta g$) in various reaction systems is being critically reexamined (Fee and Valentine, 1977; Harrison *et al.*, 1978) and more information is required before an assessment of the role of O_2 ($^1\Delta g$) in this reaction system can be made.

It appears reasonable to suggest that both O_2^- and H_2O_2 are generated in cheek pouch tissues by endogenous substrate–xanthine oxidase interactions which result in OH· and possibly other oxidizing agent generation.

An important question to be considered is: does the amount of O_2^- generated on the surface of the cheek pouch have physiological significance? Superoxide anion radical generated by the xanthine oxidase systems used during our experiments appear to be in the range estimated by Babior *et al.* (1975) for the O_2^- production by large numbers of activated polymorphonuclear leukocytes.

Role of O_2^-, H_2O_2, and OH·

The aim of this study was to investigate the role played by O_2^-, H_2O_2, and OH· in the permeability changes observed after topical application of xanthine oxidase to the cheek pouch. Since xanthine oxidase inhibition would result in a decreased flux of both O_2^- and H_2O_2, *in vitro* experiments were performed to eliminate enzyme inhibition as a mechanism of action of the substances used. SOD, CAT, DMSO, and L-methionine at the concentrations employed did not inhibit *in vitro* xanthine oxidase function as determined by uric acid generation from hypoxanthine (Table 3). If these *in vitro* results can be extrapolated to endogenous cheek pouch substrate–xanthine oxidase interactions then enzyme inhibition is unlikely to account for the influence of these substances. The products of these enzyme–substrate reactions should not be decreased by the substances tested and may be increased in the presence of L-methionine (Table 3). The O_2^- generation rate was significantly decreased only by the concentrations of SOD employed (Table 3). SOD functions as an enzymatic scavenger of O_2^- by catalyzing reaction (3) (McCord and Fridovich, 1969). The influence of the other substances employed can not be attributed to a reaction with O_2^-.

Addition of either 50 μg/ml SOD or 50 μg/ml CAT to the reservoir fluid bathing the cheek pouch prior to addition of xanthine oxidase resulted in a significant decrease in leakage sites per square centimeter (Figs. 1–3). These results suggest

that O_2^- and H_2O_2 are not the primary agents responsible for the increased macromolecular extravasation. Extracellular SOD concentrations are low relative to intracellular concentrations (McCord, 1974; Salin and McCord, 1977) and exogenous addition of SOD would result in a greater percentage of generated O_2^- being further reduced to H_2O_2 (reaction (3)). Since the presence of 50 μg/ml SOD would increase H_2O_2 while decreasing macromolecular extravasation it suggests that H_2O_2 alone is not responsible for the permeability changes. Similarly the presence of 50 μg/ml CAT although removing H_2O_2 would not decrease O_2^- generation implying no direct role for O_2^-. *In vitro* tests demonstrated no significant decrease in O_2^- generation in the presence of 50 μg/ml CAT and therefore any contamination of CAT with SOD (Halliwell, 1973) would not be expected to influence results. SOD in the concentrations employed did not react with H_2O_2 and therefore contamination of SOD with CAT is unlikely. The failure of SOD 10 μg/ml to significantly decrease leakage although decreasing O_2^- generation rate in the *in vitro* system (Table 3) may be related to the necessity of close proximity of SOD to the endogenous substrate–xanthine oxidase reactions which may not have been obtained at this concentration.

Although O_2^- and H_2O_2 are not themselves the active agents, the presence of both is necessary because removal of either resulted in decreased macromolecular extravasation. These two molecular species may interact as described previously to generate OH· which may be the active agent.

To test this concept DMSO, an OH· scavenger (Dorfman and Adams, 1973) and L-methionine which reacts with both OH· (Dorfman and Adams, 1973) and O_2 ($^1\Delta g$) (Bellus, 1978) were used. These compounds were selected since they did not significantly decrease *in vitro* O_2^- generation rate (Table 3) or react with H_2O_2, and were very effective in 10 m*M* concentrations in other model systems (unpublished results). The addition of either 10 m*M* DMSO or 10 m*M* L-methionine resulted in a significant decrease in leakage sites per square centimeter (Fig. 4). These results support the concept that OH· may be directly involved in the initiation of macromolecular extravasation. L-Methionine was the most effective of the agents used. The rate constants for the reaction of DMSO and L-methionine with OH· are similar (Dorfman and Adams, 1973), so the difference in their effectiveness can not be related only to their ability to react with OH·. The amount of O_2 ($^1\Delta g$) production during endogenous substrate–xanthine oxidase reactions is unknown. However, the increased effectiveness of L-methionine could be related to its ability to quench any O_2 ($^1\Delta g$) produced.

In the cheek pouch model endogenous substrate–xanthine oxidase reactions may occur with the extracellular generation of O_2^- and H_2O_2 which subsequently form OH· as diagrammatically presented in Fig. 5. This emphasizes that in the presence of low extracellular concentrations of SOD, O_2^- may undergo spontaneous dismutation to H_2O_2 or reduce chelated metal complexes present in the interstitial space and/or buffer. In the presence of reduced metal complexes and low extracellular catalase levels (McCord, 1974) the H_2O_2 formed will generate OH·. Hydroxyl radicals once formed in the extracellular space can react either directly or through other radical intermediates, such as the carbonate anion and formate radicals (Michelson, 1977), with the polyunsaturated fatty acids of the plasmalemma. Lipid peroxide radical formation (ROO·) can occur with the subsequent abstraction of hydrogen from a neighboring fatty acid initiating chain

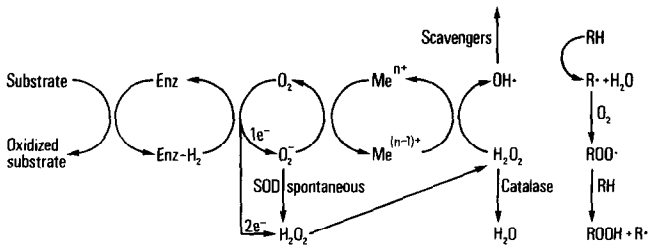

FIG. 5. Proposed scheme for the election flux in the enzyme system studied. The electron flow is from substrate to xanthine oxidase (Enz–H_2) which reduces O_2 univalently ($1e^-$) to O_2^- or divalently ($2e^-$) to H_2O_2. The O_2^- generated may reduce metal chelates (Me^{n+}) to the reduced form ($Me^{(n-1)+}$) or dismutates spontaneously to H_2O_2. The H_2O_2 formed may react with $Me^{(n-1)+}$ by a Fenton reaction to generate OH·. This molecule may react with a variety of compound including lipids to generate other radicals (R·). In the case of lipids, O_2 may react with R· to form lipid peroxide radicals (ROO·) which, by abstracting a hydrogen, can initiate chain reactions and form lipid hydroperoxides (ROOH) which may be involved in endothelial cell alterations. SOD protection results from its ability to dismutate O_2^- and therefore prevent reduction of Me^{n+}. Since $Me^{(n-1)+}$ is not formed, even excessive H_2O_2 cannot generate OH· and less tissue injury results, catalase by catalytically decomposing H_2O_2 to H_2O prevents OH· production by removing H_2O_2, the substrate for the Fenton reaction. DMSO and L-methionine by scavenging OH· preferentially would decrease its interaction with other cellular components.

reactions and formation of hydroperoxides (ROOH). Two major consequences can result from these self-propagation radical chain reactions. The first is that large regions of plasmalemmal membrane may undergo peroxidative pertubation which can result in the cell's inability to maintain its internal milieu. This may lead to disruption of the plasmalemma and release of intracellular components such as lysosomal enzymes which can further damage tissue (Fong *et al.*, 1973). Second, various lipid hydroperoxides and other fatty acids may be released into the interstitial space. Hydroxyl radical or a generated product may also influence phosopholipase function causing the release of arachidonic acid from lipid membranes and generation of endoperoxide metabolites (Pryor and Stanley, 1975). During experiments in which arachidonic acid (unpublished results) was added to the hamster cheek pouch a dose-dependent increase in leakage per square centimeter was seen which was not observed when linoleic acid was added. The pretreatment of animals both intravenously and directly to the surface of the pouch with indomethacin resulted in some decrease in macromolecular extravasation. However, at high concentrations no benefit was observed suggesting a cyclooxygenase-independent mechanism for the increased permeability seen, possibly mediated by hydroperoxides. It would appear that if OH· interaction with lipid membranes released hydroperoxides or arachidonic acid, these and other metabolites could be generated which may influence endothelial cell contraction resulting in macromolecular leakage. Injury to human endothelial cells in culture can be induced by a xanthine–xanthine oxidase system which can be decreased by a combination of both SOD and CAT (Sacks *et al.*, 1978). Although OH· scavengers were not tested it suggests that OH· may also cause direct endothelial cell injury.

The results of addition of the substances used can be explained in the light of these considerations (Fig. 5). SOD by enzymatically scavenging O_2^- would prevent O_2^--mediated reduction of chelated metal complexes and although H_2O_2

concentration would increase, OH· formation would not result. The presence of CAT would remove H_2O_2 formed and little H_2O_2 would remain to generate OH·. DMSO and L-methionine by their ability to scavenge OH· directly decreased its ability to further generate active products.

Free Radicals and Inflammation

The inflammatory process is characterized by a decreased integrity of the endothelial cell barrier, vascular alterations, and the presence of inflammatory cells. The interrelationships among these changes are very complex and the role of O_2-derived free radicals and their products is beginning to be appreciated (Salin and McCord, 1977). The activation during the inflammatory process of polymorphonuclear leukocytes, macrophages, and monocytes (Babior, 1978) by a variety of stimuli results in the release of O_2^- into the extracellular space (Root and Metcalf, 1977). The low extracellular SOD and CAT concentrations allows both the formation and accumulation of H_2O_2 and O_2^-mediated reduction of metal chelates resulting in OH· formation (Fig. 5). Hydrogen peroxide is also a substrate for the myeloperoxidase reaction which generates further oxidative products such as hypochlorite (Klebanoff, 1975) and possibly O_2 ($^1\Delta g$) (Rosen and Klebanoff, 1979). These oxidative components derived from the ability of inflammatory cells to release O_2^- provides them with an effective bactericidal arsenal (Babior, 1978). The oxidative species are not specific and may degrade structural molecules such as hyaluronic acid (McCord, 1974; Halliwell, 1978; Del Maestro *et al.*, 1978), result in peroxidative membrane disruption (Kellogg and Fridovich, 1977), and possibly release and generate active lipid products. These events may contribute to the permeability alterations seen during inflammation. A complicated relationship exists between H_2O_2 and other radical species and prostaglandin synthesis. CAT and OH· scavengers decrease prostaglandin formation *in vitro* while superoxide dismutase is without effect or may increase prostaglandin production (Panganamala *et al.*, 1974). These results would suggest that O_2^- may not play a role in prostaglandin generation but that H_2O_2 and OH· are involved. A dissociation between prostaglandin synthesis and lipid peroxidation was seen in the kaolin-induced granuloma pouch model in rats (Bragt *et al.*, 1979). It would appear that the role of free radicals during inflammation cannot be explained only by their role in prostaglandin formation and we suggest that lipid hydroperoxides released from plasma membranes by OH· may also be important mediators of the inflammatory response.

A better understanding of the interrelationships between O_2^--derived free radicals released from inflammatory cells and the other biochemical and pathological components of inflammation should provide new avenues for treatment. SOD is presently being tested in various inflammatory conditions (Meander-Huber and Huber, 1977). CAT influence is less clear although it appears to decrease edema in an inflammatory model (Ohmori *et al.*, 1978) and reduces burn-induced plasma volume loss in dogs (Hilton, 1973). DMSO has previously been widely used as an anti-inflammatory agent (Wood and Wood, 1975).

Our results suggest that some of the permeability increase seen during inflammation may be related to the release of O_2^- from inflammatory cells possibly mediated through the interactions of OH· with plasmalemmal fatty acids. Furthermore, the function of SOD, CAT, and OH· scavengers may be to inhibit these changes. In this regard, our findings may also be relevant to the under-

standing of endothelial cell injury which occurs in a wide variety of disease states. The lung injury associated with hyperoxygenation, shock, and paraquat toxicity are all associated with a high-protein pulmonary edema and aggregations of inflammatory cells. An assessment of the role of individual radical species in these and related conditions may be useful in developing new approaches to their understanding and treatment.

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