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Increase in Microvascular Permeability Induced by Enzymatically Generated Free Radicals

I. *In Vivo* Study[1]

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The inflammatory process is associated with the activation of phagocytic cells such as polymorphonuclear leukocytes with the subsequent release and generation into the extracellular space of a group of active compounds, some of which are free radicals. The relationship of these radical species to the other features of inflammation is unknown. The aim of this study was to assess the influence of free radicals generated by the aerobic oxidation of hypoxanthine by xanthine oxidase on the *in vivo* microvascular preparation of the hamster cheek pouch. Fluorescein-labeled Dextran, $\bar{M}_w$ 150,000, was used to assess microvascular permeability. The application of xanthine oxidase alone or hypoxanthine and xanthine oxidase resulted in a significant increase in macromolecular extravasation which was not seen after addition of hypoxanthine, uric acid, or denatured enzyme. Increased leukocyte rolling and arteriolar vasoconstriction were also observed. The addition of xanthine oxidase to cheek pouch homogenates, *in vitro*, demonstrated that intrinsic cheek pouch substrates were available for enzyme-induced generation of superoxide anion radical. These results suggest that xanthine oxidase acting on exogenous and/or endogenous substrates generates a flux of free radicals which may further interact to result in the increased macromolecular permeability seen. It is suggested that the permeability changes seen during the inflammatory process may be in part related to the release of free radicals from inflammatory cells.

INTRODUCTION

Oxygen-derived free radicals have been implicated in a wide variety of disease states (Slater, 1972; Pryor, 1976; Fridovich, 1978), however, the precise role played by the individual reactive species and their products in normal and pathological processes remains unclear. A free radical is defined as any substance possessing an unpaired electron in its outer orbital and is generated in living

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tissue by irradiation (Benon *et al.*, 1977), homolysis (Pryor, 1966, 1967), and during oxidation–reduction reactions (Prior, 1976; Yamazaki, 1977).

Acute inflammation is associated with an increase in vascular permeability due to endothelial cell alterations with subsequent edema, polymorphonuclear leukocyte infiltration, and activation (Thorgeirsson and Robertson, 1978). An essential component of the respiratory burst of activated inflammatory cells such as polymorphonuclear leukocytes is the univalent enzymatic reduction of O_2 to the superoxide anion radical (O_2^-) (Babior *et al.*, 1973; Babior, 1978; Johnston *et al.*, 1975). A large proportion of the generated O_2^- is released into the extracellular space (Root and Metcalf, 1977) where spontaneous dismutation can occur with the production of H_2O_2 and O_2 (Fridovich, 1976, 1978; Babior, 1978). The simultaneous presence of O_2^-, H_2O_2, and chelated metal catalyst(s) in the extracellular space can result in further generation of more active oxygen derived molecules such as hydroxyl radical (OH·) and singlet O_2 ($O_2(^1\Delta g)$) (Haber and Weiss, 1934; Kahn, 1970; Beauchamp and Fridovich, 1970; Fong *et al.*, 1976; Kellogg and Fridovich, 1977; McCord and Day, 1978; Halliwell, 1978).

The primary function of O_2^- release and subsequent generation of other reactive species by activated inflammatory cells may be to aid in bacterial killing (Babior *et al.*, 1975; Babior, 1978; Rosen and Klebanoff, 1979). However, peroxidative disruption of cellular membranes (Kellogg and Fridovich, 1977) and degradation of essential structural biopolymers, such as hyaluronic acid (McCord, 1974; Halliwell, 1978; Del Maestro *et al.*, 1978), can also occur in the presence of these same molecules.

It was considered important to assess the role of the extracellular generation of free radicals on an *in vivo* microcirculatory model system to elucidate their role, if any, in the changes seen during inflammation. The hamster cheek pouch microvasculature has been extensively employed (Duling, 1973; Duling and Staples, 1976; Svensjö *et al.*, 1978; Svensjö, 1978) to assess the influence of various compounds on microvascular integrity and was used in this study. Xanthine oxidase, acting aerobically on hypoxanthine to produce uric acid, produces a flux of O_2^- and H_2O_2 (Fridovich, 1978) with the subsequent generation of OH· (Beauchamp and Fridovich, 1970) and possibly O_2 ($^1\Delta g$) (Kellogg and Fridovich, 1977). This enzyme system was used to generate these activated oxygen species both *in vitro* and on the surface of the cheek pouch. O_2^- generation was assessed and changes in the underlying microvasculature monitored by *in vivo* microscopy.

In this communication the results of the *in vivo* experiments are reported and in the accompanying paper an attempt is made to unravel the role played by the individual free radicals in the changes observed.

MATERIALS AND METHODS

Enzymes

Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2, Grade 1) was obtained from the Sigma Chemical Company, St. Louis, Missouri. Xanthine oxidase (0.66 units/mg protein) was dissolved in the superfusion solution immediately prior to application to the cheek pouch and used undiluted during *in vitro* experiments. Denatured xanthine oxidase was prepared by boiling the active enzyme at 100° for 20 min and the concentration of denatured enzyme used is expressed as the number of active units denatured.

Chemicals

Hypoxanthine and cytochrome c^{3+} (Type VI) were obtained from Sigma Chemicals while uric acid and the buffer salts were from E. Merck, Darmstadt, West Germany.

Solutions

The cheek pouch superfusion fluid was a bicarbonate-buffered saline solution (Duling and Staples, 1976) with the composition (millimolar): NaCl, 131.9; KCl, 4.7; $CaCl_2$, 2.0; $MgSO_4$, 1.2; and $NaHCO_3$, 18. All solutions used for either *in vivo* or *in vitro* studies were prepared in the bicarbonate buffer bubbled with 5% CO_2 and 95% N_2 to maintain the P_{O_2} *between 2–4* pK_a, a pH of 7.35, and warmed to 37°.

Preparation of Animals

Male golden hamsters (*Mesocricetus auratus*) weighing 70–120 g (Stockholm Biologiska Laboratorium) were anesthetized with 60 mg/100 g sodium pentobarbital intraperitoneally (Nembutal, Abbott, Chicago, Ill.). A tracheostomy was performed and a P_{10} (Portex) catheter placed into a femoral vein for administration of supplemental doses of anesthesia and the tracer substance. The cheek pouch was prepared according to Duling (1973) with modifications (Svensjö *et al.*, 1978) for intravital microscopy of macromolecular permeability. The hamster and prepared pouch were transferred to the stage of a Leitz Ortholux microscope where the cheek pouch could be continuously superfused, the superfusion fluid and hamster body temperature monitored and maintained at 37°. FITC–Dextran 150 (fluorescein-labeled dextran $\bar{M}_W$ = 150,000, Pharmacia, Uppsala, Sweden) was administered intravenously (25 mg/100 g) in a 5% solution in 0.9% NaCl.

Microscopy

The cheek pouch was observed through a Leitz Ortholux microscope, transilluminated with a 100-W Hg dc lamp (Irem Model EIXH5 P/L) with observations and micrographs taken at 35× magnification. Micrographs were taken of some regions assessed on Ektachrome 160 Tungsten developed for the exposure of 800 ASA.

Permeability Studies

The cheek pouches were observed for a 60-min equilibration period following FITC–Dextran 150 injection. Macromolecular leakage sites were observed as fluorescent regions in the interstitium originating from the vessel extravasating the intravascular tracer (Fig. 2). The number of leakage sites per square centimeter counted represents a semi-quantitative assessment of the degree of macromolecular extravasation (Svensjö *et al.*, 1978). Leakage sites were counted at 10-min intervals prior to application of test solution(s). The application sequence was randomized and test solution(s) applied to the cheek pouch of each animal using standardized procedures.

Procedure 1. The superfusion was discontinued and the solution carefully removed from the microscopic stage reservoir. This was replaced by 10 ml of freshly prepared solutions of either (i) bicarbonate buffer, (ii) 0.96 m*M* hypoxanthine, or (iii) 0.96 m*M* uric acid.

Procedure 2. Superfusion was discontinued and a 1.0-ml solution of denatured

xanthine oxidase or 1.0 ml of active xanthine oxidase was added to the surface of the cheek pouch to give a final concentration in the reservoir of 0.05 units/ml enzyme.

Procedure 3. Superfusion was stopped and the reservoir pool replaced with 0.96 m*M* hypoxanthine to which was added 1.0 ml of active xanthine oxidase solution.

In all experiments the solution(s) were left in contact with the cheek pouch for 1 min then removed by recommencing the superfusion. The cheek pouch area was scanned and leakage sites counted at 5, 10, 15 min, and thereafter at 10-min intervals for a total experimental observation time of 85 min.

Assay of Superoxide Generation

The reduction of cytochrome c^{3+} (Cyt c^{3+}) by O_2^- was assayed in an *in vitro* system composed of 0.96 m*M* hypoxanthine, 50 μ*M* Cyt c^{3+} in bicarbonate buffer to which xanthine oxidase was added at a concentration of 0.05 units/ml in a final volume of 2.5 ml. The increase in absorbance at 550 nm was monitored at 37° with a double-beam spectrophotometer (Model 124, Hitachi, Perkin–Elmer, Norwalk, Conn.) equipped with 1-cm thermostatted curvettes ($\Delta\varepsilon_{550}$ = 21,000 M^{-1} cm^{-1}) (Massey, 1959).

Tissue homogenates were prepared from eight hamster cheek pouches which had not been subjected to any test procedures. The cheek pouches were freeze-dried (Mini-Fast, Mod 1700; Edwards, Sussex, England), suspended in bicarbonate buffer (0.5 ml buffer/10 mg freeze-dried pouch), and homogenized. The supernatant was obtained after centrifuging for 20 min at 2000 rpm. Superoxide radical generation was assessed in a solution containing 50 μ*M* Cyt c^{3+}, 1.2 ml of supernatant with and without 0.05 units/ml of xanthine oxidase in a total volume of 2.5 ml. The initial O_2^- formation rate was calculated as the difference between Cyt c^{3+} reduction with and without the presence of xanthine oxidase.

Statistical Methods

Measurements quoted are mean values + SEM. Statistical significance was calculated using the rank–sum test and a value of $\alpha < 0.025$ was considered significant (Dixon and Massey, 1957).

RESULTS

FITC–Dextran 150 appeared in all cheek pouch preparations 8–20 sec following intravenous administration and within 35 sec was observed in all vessels of the microvascular bed. Using fluorescent microscopy and 35× magnification the hamster cheek pouch microvascular architecture can be clearly outlined (Fig. 1).

Table 1 shows the number of leakage sites per square centimeter seen before and after application of the test solution(s) to the hamster cheek pouch. During the 60-min equilibration period the number of leakage sites seen varied between individual preparations. However, no statistically significant differences were noted among the various groups prior to test solution(s) application. Discontinuation of superfusion and replacement of the reservoir fluid with bicarbonate buffer for 1 min followed by recommencing superfusion was associated with the

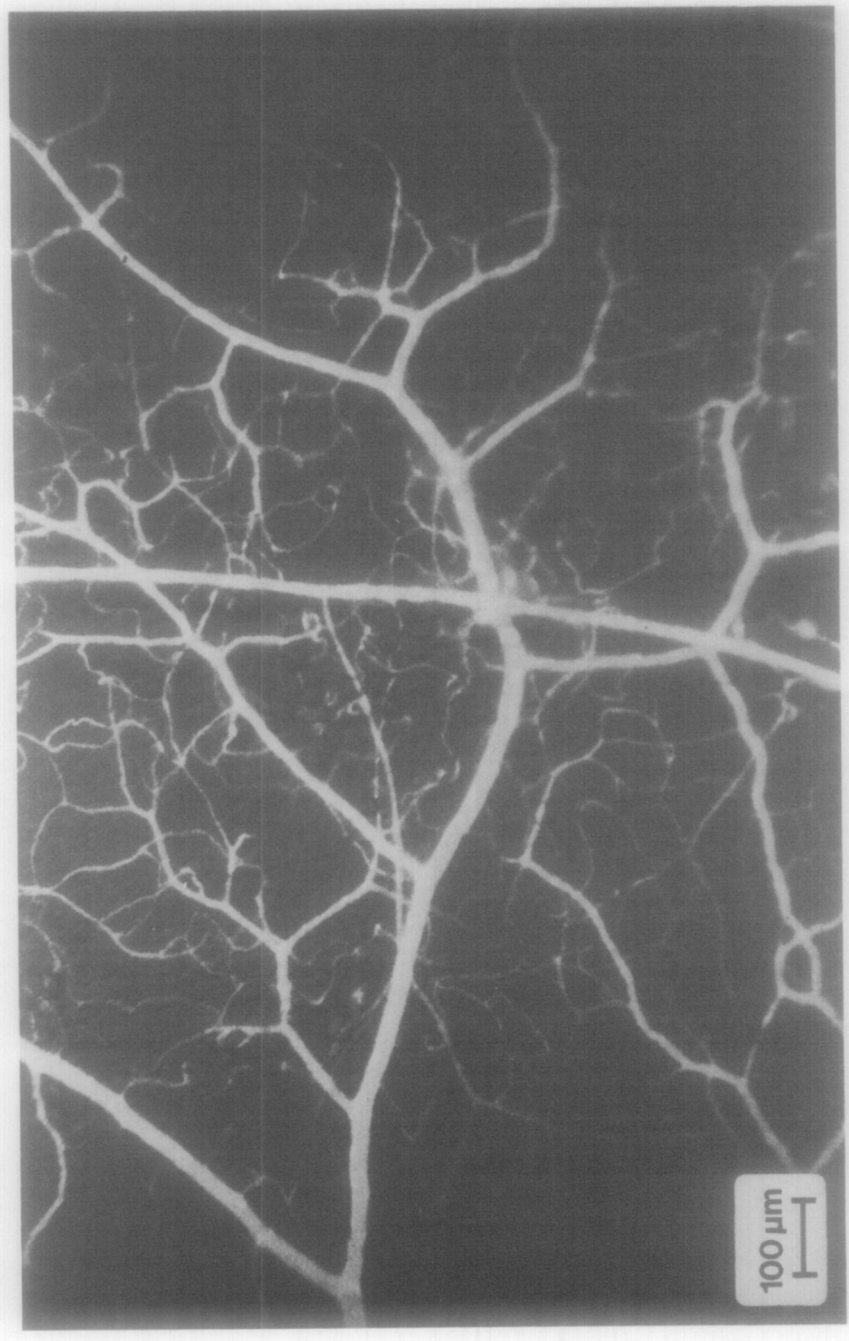

FIG. 1. **Micrograph of hamster cheek pouch microvasculature taken in fluorescent light at 35× magnification prior to application of 0.96 m*M* hypoxanthine and 0.05 unit/ml of active enzyme.**

TABLE 1
EFFECT OF TOPICAL APPLICATION OF TEST SOLUTION(S) ON MACROMOLECULAR LEAKAGE SITES[a]

Time after application (min)	Test solution: Bicarbonate buffer	Hypoxanthine (0.96 m*M*)	Uric acid (0.96 m*M*)	Denatured xanthine oxidase (0.05 unit/ml)	Xanthine oxidase (0.05 unit/ml)	Hypoxanthine (0.96 m*M*), xanthine oxidase (0.05 unit/ml)
Preapplication	1.5 ± 0.9	3.2 ± 2.7	3.4 ± 1.6	0.0 ± 0.0	1.5 ± 1.2	2.2 ± 1.1
5	2.5 ± 1.6	4.8 ± 2.8	4.9 ± 2.0	0.7 ± 0.5	107.9 ± 38.2*	207.9 ± 64.0*
10	1.7 ± 1.1	5.7 ± 3.3	5.1 ± 2.8	1.0 ± 0.6	265.4 ± 36.7*	295.6 ± 37.0*
15	2.0 ± 1.1	10.1 ± 7.3	4.4 ± 2.6	0.5 ± 0.3	212.5 ± 54.8*	238.3 ± 44.4*
25	2.0 ± 1.1	10.3 ± 7.5	3.7 ± 2.2	0.0 ± 0.0	127.7 ± 52.2*	164.6 ± 44.8*
35	2.5 ± 0.8	11.8 ± 8.2	4.5 ± 2.3	0.5 ± 0.5	103.7 ± 52.6	134.6 ± 42.4*
45	1.7 ± 1.0	6.6 ± 4.3	4.7 ± 2.8	0.7 ± 0.5	80.4 ± 39.4*	93.7 ± 28.5*
55	2.0 ± 1.1	5.7 ± 3.6	5.4 ± 3.2	1.5 ± 1.0	61.1 ± 31.6*	77.2 ± 28.0*
65	2.0 ± 1.2	6.4 ± 4.1	5.3 ± 3.5	2.2 ± 1.3	52.0 ± 25.4	90.2 ± 53.1*
75	2.5 ± 1.6	6.6 ± 4.2	6.9 ± 4.3	2.2 ± 1.1	44.1 ± 18.9	89.0 ± 39.7*
85	4.7 ± 3.8	6.6 ± 4.3	8.8 ± 5.9	2.5 ± 1.7	38.7 ± 15.2	89.7 ± 40.8*

[a] Values are mean number of leakage sites/cm^2 ± SEM; $n = 6$ for all test solutions. Leakage site values were compared in each group at each time period to bicarbonate buffer as a control using the rank-sum test. Significant difference is indicated (*) when $\alpha < 0.025$.

appearance of occasional new leakage sites. However, the flow remained normal throughout the preparation and only a small number of new leaks appeared during the 85-min observation period. Solutions containing 0.96 m*M* hypoxanthine or 0.96 m*M* uric acid were associated with no observable alterations in the microvascular vessels and no statistically significant increase in the mean number of leakage sites per square centimeter as compared to bicarbonate buffer alone.

The application of active xanthine oxidase alone or 0.96 m*M* hypoxanthine and active enzyme was associated with a marked increase in the number of leakage sites while application of denatured enzyme caused no leakage site increase. The mean number of leakage sites observed after application of hypoxanthine and active enzyme was greater at each time interval than that observed with xanthine oxidase application alone. In both groups leakage sites usually occurred within 1–2 min of application reached a maximum at 10 min and then decreased steadily during the observation period. The decrease in leakage sites occurred more rapidly in the xanthine oxidase-alone group. In both groups large areas of cheek pouch were without observable leakage sites during the last 30 min of the observation period.

Figures 1–4 are typical micrographs taken prior and following application of 0.96 m*M* hypoxanthine and active xanthine oxidase to the reservoir surrounding the cheek pouch. Figure 1 demonstrates the hamster cheek pouch microvasculature showing no leakage sites immediately prior to application, while Fig. 2 clearly shows macromolecular extravasation from postcapillary venules 2 min later. In Fig. 3 the same region is shown 12 min following application with evidence of continuing macromolecular leakage with extravasation appearing to originate from larger venules (>20 μm) and Fig. 4 demonstrates complete resolution of macromolecular extravasation at 60 min.

The majority of the leakage sites observed were associated with postcapillary venules and larger venules. Macromolecular extravasation was not observed from arteries, arterioles, or capillaries. It was difficult to differentiate macromolecular extravasation associated with larger venules from the flowing together of multiple postcapillary leakage sites giving the false impression of direct venular leakage. However, at times, extravasation could be seen originating directly from a larger venule (Fig. 5) in the absence of any associated postcapillary extravasation which confirmed that macromolecular extravasation did indeed occur from these vessels. In two of six cheek pouches to which hypoxanthine and active enzyme was added petechial hemorrhages (Fig. 6) were seen at 50 and 80 min. These occurred in regions in which macromolecular leakage continued unabated during the observation period.

The applications of active enzyme or active enzyme and hypoxanthine were associated with initial arteriolar vasodilation which was followed by vasoconstriction (below initial diameter) occurring 3–5 min after addition with relaxation occurring within another 5 min. The application of denatured enzyme was also associated with the initial arteriolar vasodilation which slowly relaxed over a 10- to 15-min period with no vasoconstriction being observed. There also appeared to be increased leukocyte rolling in both arterioles and venules after application of active enzyme or active enzyme and hypoxanthine. The causes of these changes are presently under investigation.

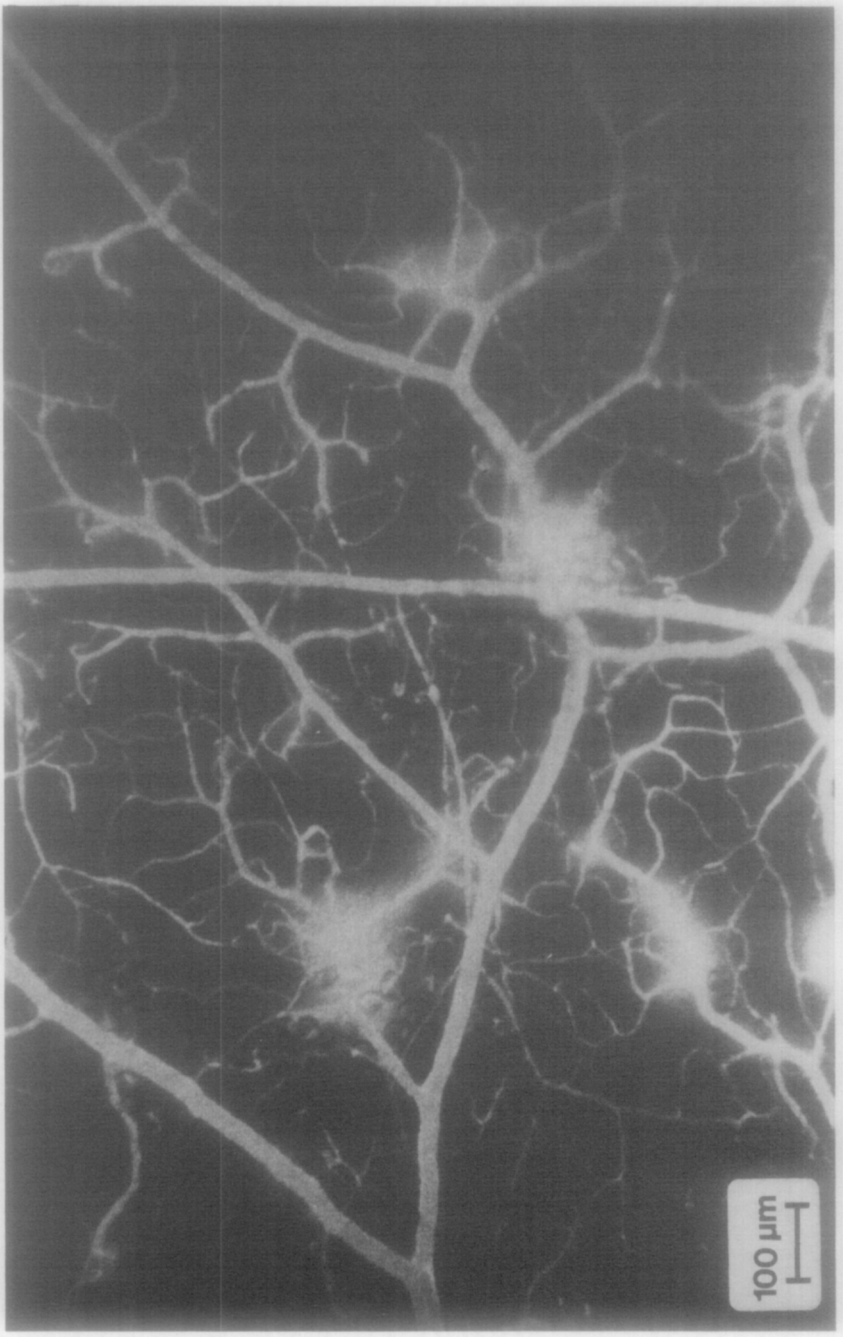

FIG 2. Same region as in Fig. 1 2 min following application of substrate and enzyme demonstrating FITC–Dextran 150 extravasation from postcapillary venules.

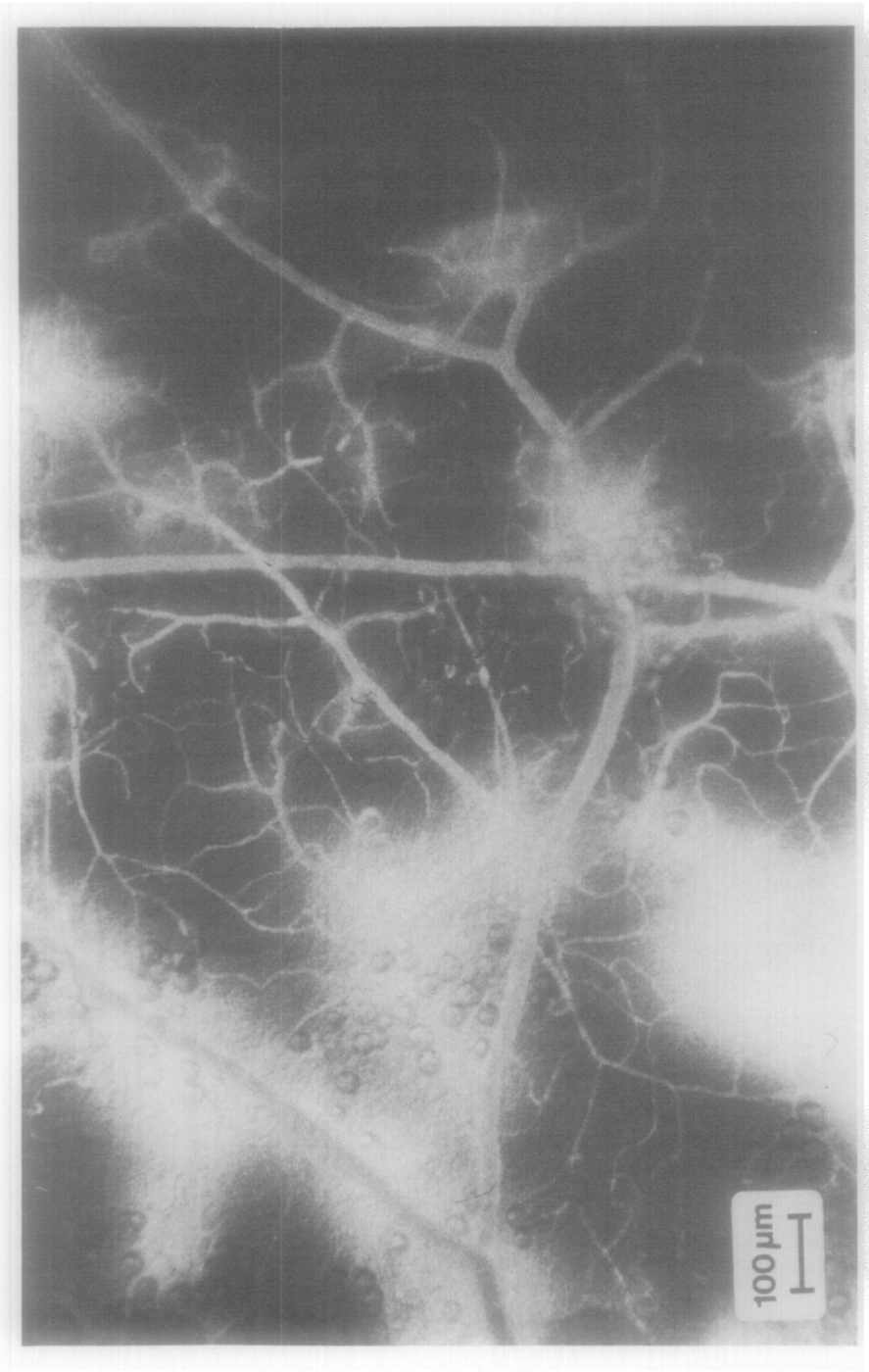

FIG. 3. **Twelve minutes following application with evidence of continuing macromolecular leakage and extravasation appearing to originate from** larger venules (20 µm).

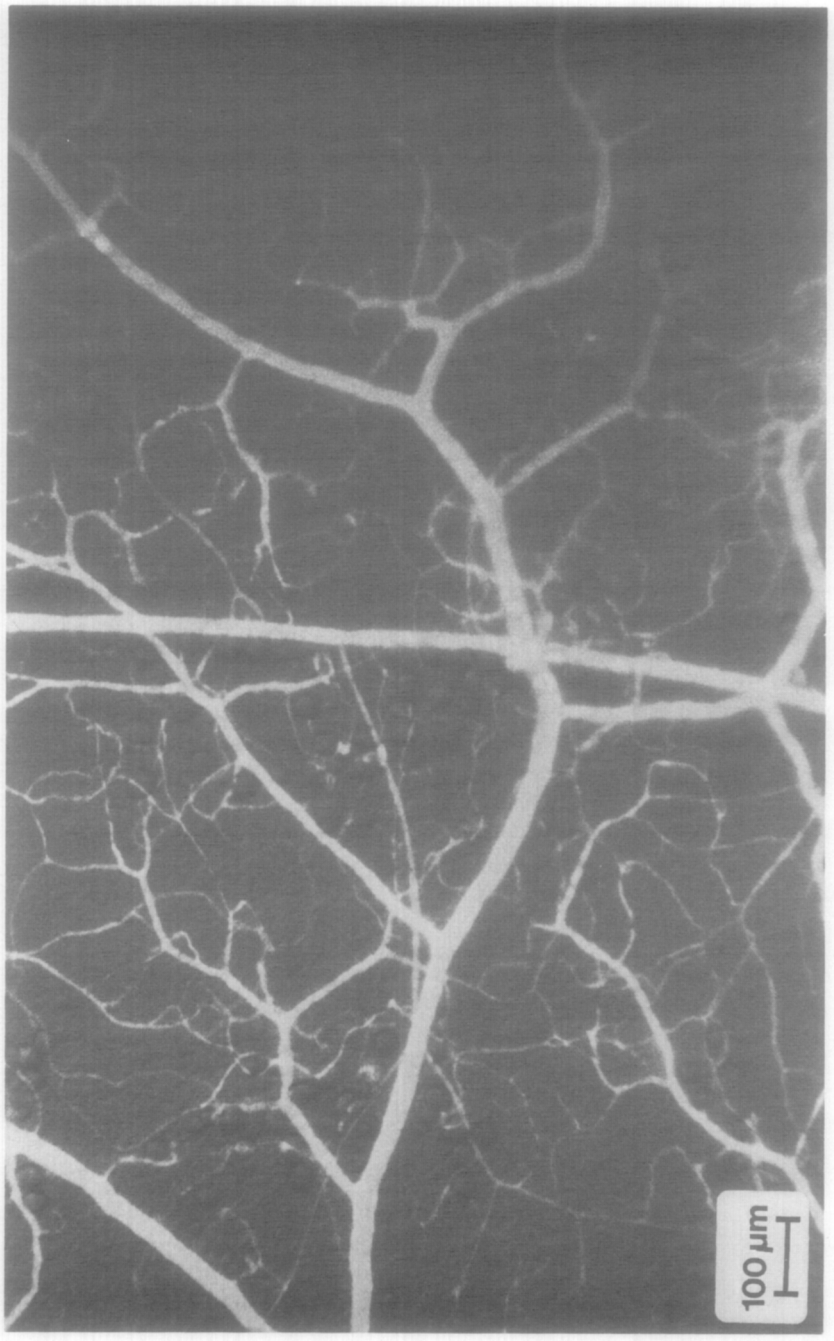

FIG. 4. Same region as in Fig. 3 demonstrating complete resolution of macromolecular extravasation at 60 min.

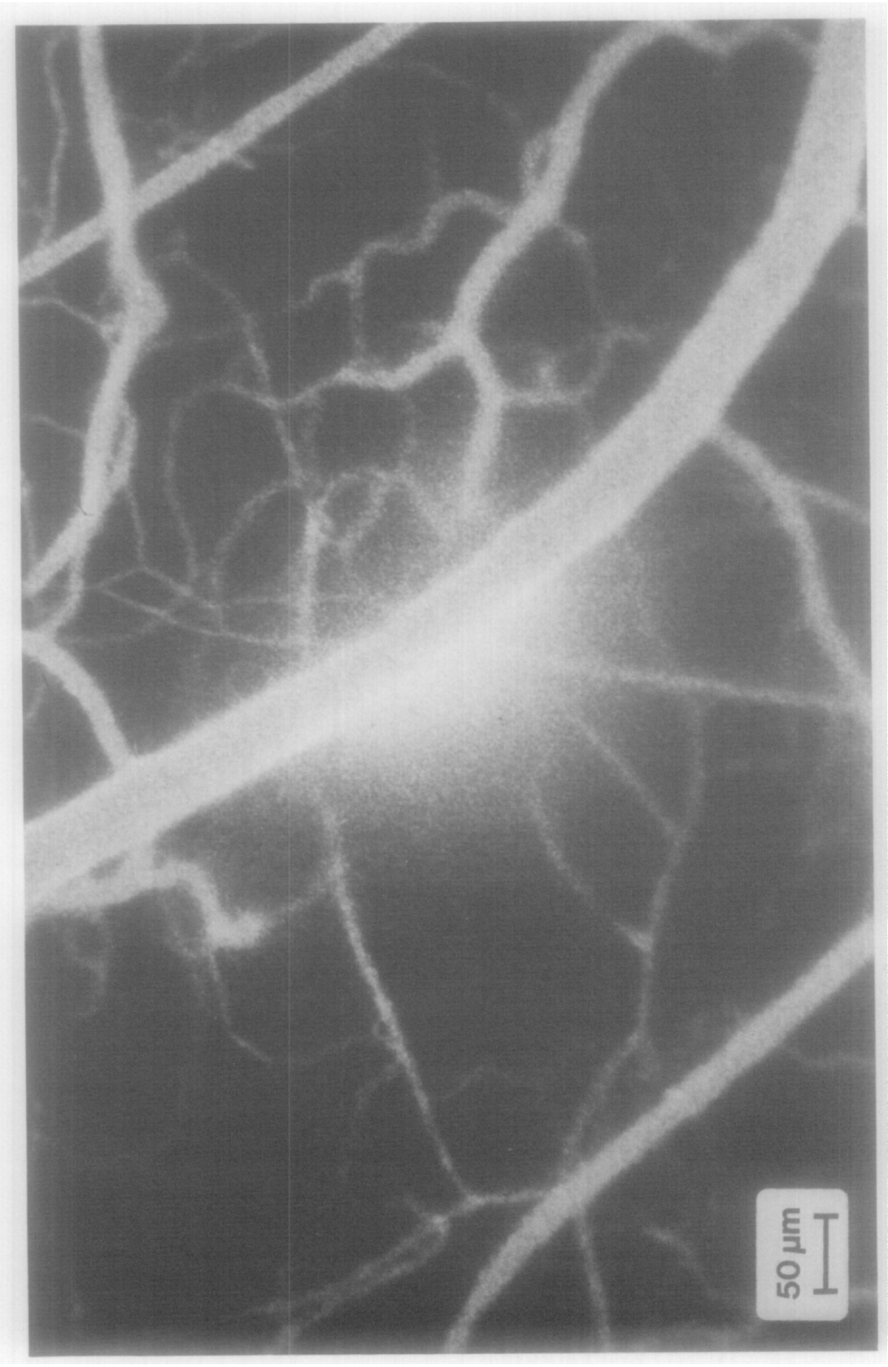

FIG. 5. Micrograph which demonstrates FITC–Dextran 150 extravasation from 50-µm vein 15 min following application of 0.05 unit/ml xanthine oxidase.

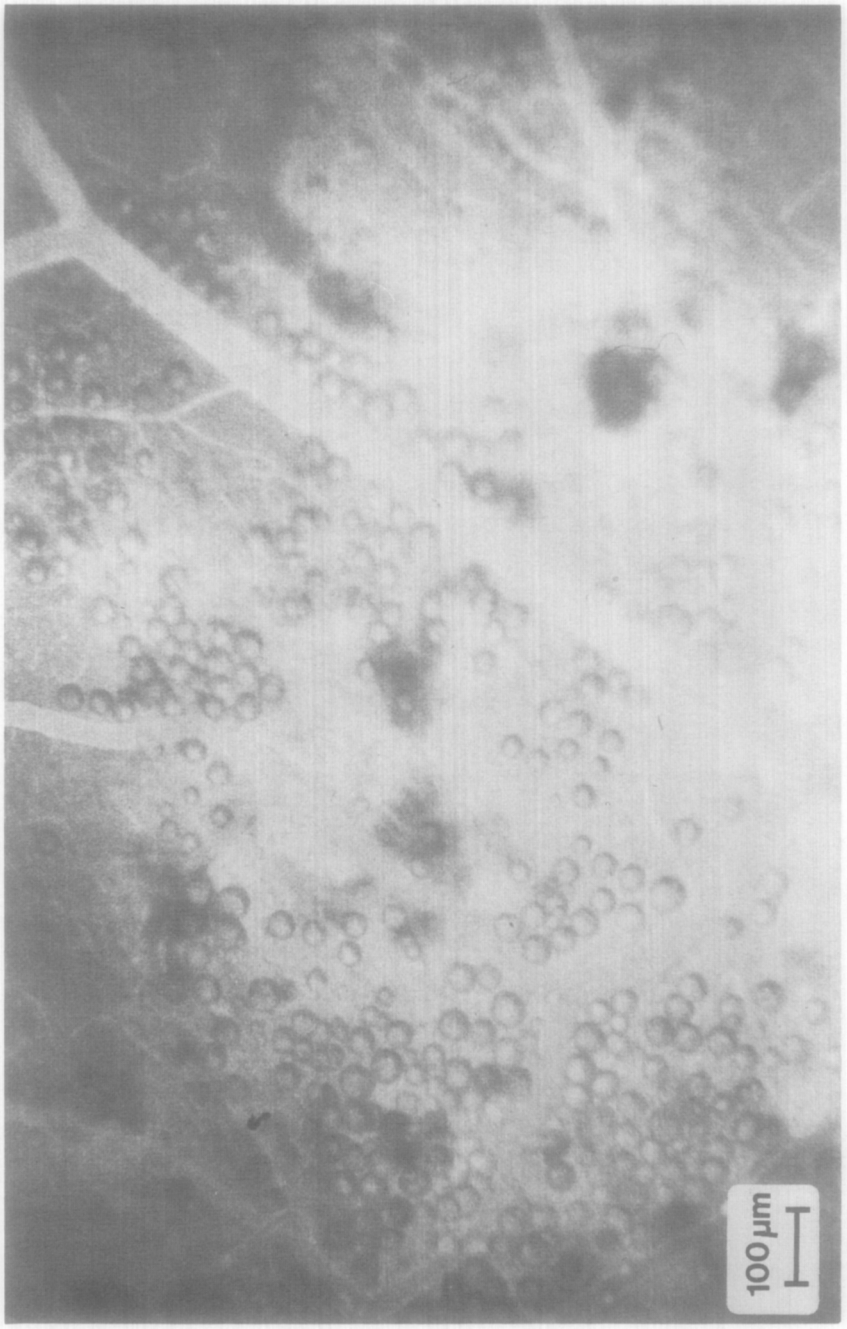

FIG. 6. Multiple petechial hemorrhages which are seen as darker regions in a region of marked extravasation of FITC–Dextran 150 50 min after application of 0.96 m*M* hypoxanthine and 0.05 unit/ml xanthine oxidase.

Since neither hypoxanthine (the substrate of the aerobic oxidation by xanthine oxidase) nor uric acid (the product of the reaction) resulted in increased macromolecular permeability it was hypothesized that the changes seen may be related to the associated free radical flux. Xanthine oxidase can act on a wide variety of compounds (Bray, 1963) and endogenous cheek pouch substances (purines, aldehydes) could provide the substrates needed for the generation of a free radical flux when exogenous substrates were omitted. Addition of active enzyme to cheek pouch homogenate was associated with an O_2^- generation rate of 4.3 ± 0.7 nmole/min/ml which is 30% of the O_2^- generation of the *in vitro* system containing hypoxanthine and active xanthine oxidase (Table 2). Denatured enzyme resulted in no measurable O_2^- formation. Some Cyt c^{3+} reduction was also seen in the absence of active enzyme and may be related to reducing agents present in the cheek pouch homogenate (Crapo *et al.*, 1978). These results confirm that endogenous substrates are available in the cheek pouch which can result in O_2^- generation on addition of active enzyme.

DISCUSSION

A spectrum of alterations in macromolecular permeability was observed after application of hypoxanthine and the enzyme, xanthine oxidase, to the cheek pouch of the hamster. Extravasation of FITC–Dextran 150 occurred from postcapillary venules, larger venules (>20 μm), and late petechial hemorrhage was evident. Exposure of the cheek pouch to xanthine oxidase alone induced less macromolecular leakage sites per square centimeter and petechial hemorrhages were not observed. In both groups arteriolar vasoconstriction and leukocyte adhesion were also noted. Hypoxanthine, the exogenous substrate, and uric acid, the product of the reaction, were not found to cause significant increases in the number of leakage sites when added to the cheek pouch. Xanthine, a reaction intermediate, did not accumulate during *in vitro* experiments (Del Maestro *et al.*, 1981) and preliminary cheek pouch studies using addition of xanthine alone did not demonstrate any differences from addition of only hypoxanthine. Activity of the enzyme appeared to be essential for these changes in macromolecular permeability since denatured xanthine oxidase was without effect.

The topical application of histamine (Svensjö *et al.*, 1973), bradykinin (Svensjö, 1978), and prostaglandins PGE_1, PGE_2, $PGF_{2\alpha}$ (Svensjö, 1978) to the hamster cheek pouch were associated with an increase in macromolecular extravasation

TABLE 2
RATE OF *IN VITRO* O_2^- FORMATION

	O_2^- formation (nmole/min/ml)
Hypoxanthine (0.96 m*M*) and xanthine oxidase (0.05 unit/ml), $n = 6$	12.0 ± 0.5
Denatured xanthine oxidase (0.05 unit/ml), $n = 6$	0
Cheek pouch homogenate and xanthine oxidase (0.05 unit/ml), $n = 8$	4.3 ± 0.7

Note. The assay mixtures contained 50 μ*M* Cyt c^{3+} in the bicarbonate buffer (pH 7.35) to which the test substances were added. Values represent means ± SEM for O_2^- generation rates calculated as described under Materials and Methods and *n* represents the number of experiments.

from postcapillary venules. However, extravasation from larger veins and petechial hemorrhages was not observed suggesting that the mechanism of action of activated O_2 species cannot be explained only by induced histamine and bradykinin release or by the formation of the prostaglandins mentioned.

It was hypothesized that the changes observed were related to the flux of free radicals induced by the oxidation of exogenous and/or endogenous substrates by xanthine oxidase. An essential prerequisite for this concept was that hypoxanthine and xanthine oxidase addition under the experimental conditions did in fact generate free radicals. The demonstration that O_2^- was released both *in vitro* (Table 2) and on the surface of the cheek pouch (Del Maestro *et al.*, 1981) was consistent with the hypothesis. The finding that xanthine oxidase added to the cheek pouch homogenates resulted in O_2^- production (Table 2) provides an explanation for the macromolecular leakage seen in the absence of exogenous substrate. The plantar injection of xanthine oxidase into rats is associated with foot edema which can be increased by the simultaneous presence of hypoxanthine (Ohmori *et al.*, 1978). It has also been shown that bacteria can be killed as effectively by xanthine oxidase alone as by purines and xanthine oxidase (Babior *et al.*, 1975). Xanthine oxidase, an enzyme of broad specificity (Bray, 1963), may react with endogenous substrates present in the cheek pouch and in other tissues to produce a flux of free radicals.

The plasmalemma of mammalian cells contains large amounts of polyunsaturated fatty acids (Rouser *et al.*, 1968) which can undergo oxidative change (Mead, 1976). Free radical generation by the xanthine oxidase-generating system (Kellogg and Fridovich, 1975, 1977; Thomas *et al.*, 1978) results in lipid peroxidative chain reactions with subsequent disruption of both liposomal and cellular membranes (Kellogg and Fridovich, 1977; Goldstein and Weissmann, 1977). Enzymatically generated free radicals on the surface of the cheek pouch may also initiate lipid peroxidative chain reactions within cellular membranes with the release of fatty acid hydroperoxides. The presence of these lipid peroxides and possibly others such as the endoperoxide products of arachidonic acid (Kuehl *et al.*, 1977) may result in both reversible and irreversible endothelial cell alterations. These associated with radical-induced degradation of vessel wall components, such as hyaluronic acid (McCord, 1974; Halliwell, 1978; Del Maestro *et al.*, 1978), may result in local disruption of the endothelial cell barrier with subsequent hemorrhage. The delayed appearance of the petechial hemorrhage is suggestive of a continuing process of cellular and vessel injury. The timing of the hemorrhages is reminiscent of observations in which erythrocytes begin to lyse 1–2 hr after exposure to a xanthine oxidase radical-generating system (Kellogg and Fridovich, 1977).

The generation of O_2^- in plasma by a xanthine oxidase system is associated with the formation of chemotaxic factor (McCord, personal communication). The same or a similar factor may also be responsible for the increased leukocyte rolling and adhesion observed in the present study. Lipid peroxidative change in the endothelial cell membrane may also play a role, possibly in association with the production or release of the chemotaxic factor, as a signal or marker which can be recognized by polymorphonuclear leukocytes.

The aerobic oxidation of hypoxanthine to uric acid and the respiratory burst of phagocytosing leukocytes release similar activated O_2 species (Babior *et al.*,

1975; Rosen and Klebanoff, 1979). It seems reasonable, therefore, to draw a parallel between the results of extracellular enzymatic generation of active oxygen species in the hamster *in vivo* model system and the effects of the same activated oxygen species released by inflammatory cells aggregated at sites of inflammation.

These findings suggest that O_2-derived free radicals enzymatically generated on the hamster cheek pouch are associated with increased macromolecular permeability, arteriolar vasoconstriction, and increased leukocyte rolling. The permeability changes and resultant edema seen during the inflammatory response may be related, in part, to the extracellular release of O_2^- and subsequent generation of activated oxygen species released from inflammatory cells.

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