EFFECTS OF THE GENERATION OF SUPEROXIDE ANION ON PERMEABILITY OF LIPOSOMES

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SUMMARY: Multilamellar liposomes released previously sequestered chromate ions when these artificial membrane structures were suspended in reaction mixtures containing hypoxanthine and xanthine oxidase. Release of chromate was reduced significantly if active superoxide dismutase and/or catalase were added to these reaction mixtures. These findings suggest that superoxide anion and/or related reactive molecules are capable of perturbing lipid bilayers sufficiently to cause leakage of relatively impermeant anions.

INTRODUCTION

Superoxide anion ($O_2^-\cdot$), a highly reactive radical formed by the univalent reduction of molecular oxygen, can be generated in a variety of biological systems either by auto-oxidative processes or by enzymes involved in aerobic metabolism (1). $O_2^-\cdot$ per se, as well as other reactive molecules formed as a consequence of its interaction with H_2O_2 (2, 3), have been implicated recently as being mediators of the damage to biomembranes produced by ionizing radiation (4-6) and oxidative enzymes (7-9). We have investigated the possibility that $O_2^-\cdot$ and/or related reduction products of oxygen perturb lipid bilayers sufficiently to cause leakage of relatively impermeant anions by employing as model targets for their actions, artificial dispersions of phospholipids (liposomes) containing "trapped" chromate ions. We have found that in the presence of xanthine oxidase and hypoxanthine, chromate was released from liposomes and that such leakage could be reduced significantly if superoxide dismutase and/or catalase were added to the reaction mixtures.

METHODS

Preparation of liposomes. Liposomes were prepared as described previously (10). Briefly, egg lecithin (Grand Island Biological Co., Grand Island, N.Y.), dicetyl phosphate (K & K Laboratories, Inc., Plainview,

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N.Y.) and cholesterol (Fisher Scientific Co., Fair Lawn, N.J.) were dissolved in chloroform (molar ratio 7:2:1). After rotary evaporation a uniformly thin lipid layer was formed, to which 6.0 ml of 97 mM K_2CrO_4, buffered to pH 7.3, was then added. After dispersing the lipid film by vortexing vigorously, the suspension (15 μmol lipid/ml) was allowed to stand at room temperature for 2 hours. After swelling, the suspension was filtered on a column of Sephadex G-200. Liposomes containing trapped $CrO_4^=$ were eluted with a buffer consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.3. This buffered saline was used throughout. Lipid in eluted samples was monitored by measurements of absorbance at 520 nm.

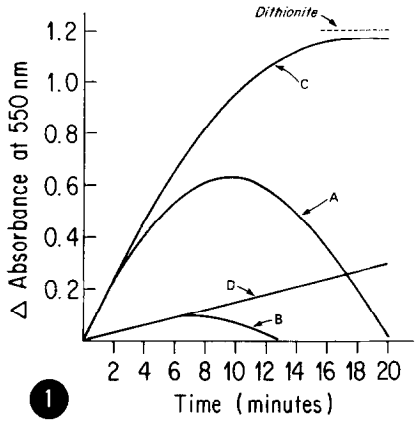
Measurement of $CrO_4^=$ release from liposomes. Aliquots (0.5 ml) of liposome suspensions (approximately 7.0 μmol lipid) were placed in small dialysis sacs containing appropriate reagents in 0.6-0.7 ml buffered saline. These reaction mixtures were dialyzed against 3.0 ml volumes of buffered saline (containing appropriate concentrations of reagents) at 22°C with constant mixing by rotation. The dialysis sacs were transferred to fresh 3.0 ml solutions at various intervals up to 24 hours. $CrO_4^=$ release was measured by its absorbance at 370 nm. Results are expressed as percent of total $CrO_4^=$ released from liposomes in suspensions to which had been added the detergent, Triton X-100 (0.2% v/v) (Rohm and Haas, Co., Philadelphia, Pa.). Release of $CrO_4^=$ from liposomes has previously been shown to parallel release of glucose, glycine or other relatively impermeant species and constitutes a convenient measure of membrane perturbation (11, 12).

Superoxide generating system. The complete system consisted of hypoxanthine (0.14 μmol) (Sigma Chemical Co., St. Louis, Mo.) and xanthine oxidase (EC 1.2.3.2) (0.013 U) (Sigma Chemical Co.). Generation of $O_2^-\cdot$ by this system was measured as described previously (13), in reaction mixtures containing 80 nmol horse heart ferricytochrome c, Type III (Sigma Chemical Co.) with and without the addition of either superoxide dismutase (EC 1.15.1.1) (Truett Laboratories, Dallas, Texas) or catalase (EC 1.11.1.6).

RESULTS

Generation of $O_2^-\cdot$ (measured as superoxide dismutase-inhibitable ferricytochrome c reduction) by the xanthine oxidase system employed in these studies is shown in Figure 1. The action of xanthine oxidase on purine substrates, under aerobic conditions, causes both univalent and divalent reductions of oxygen to yield the products, $O_2^-\cdot$ and H_2O_2 (14). These products, once formed, may react as proposed by Haber and Weiss (3) ($O_2^-\cdot + H_2O_2 \rightarrow OH^- + OH\cdot + O_2$) to yield another extremely potent oxidant, the hydroxyl radical ($OH\cdot$). That $OH\cdot$ radicals were indeed generated by the xanthine oxidase system was indicated by the delayed oxidation of ferrocytochrome c observed in reaction mixtures from which catalase was omitted (2) (Figure 1).

Liposomes suspended in buffered saline for up to 24 hours re-



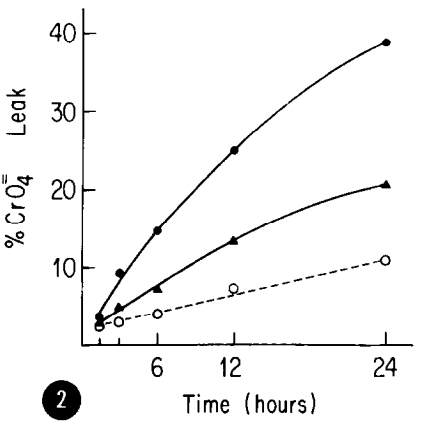


Figure 1: Cytochrome c reduction mediated by $O_2^-\cdot$ (3.0 ml buffered saline, pH 7.3, 22°C): A) hypoxanthine (0.14 μmol), xanthine oxidase (0.013 U), and cytochrome c (80 nmol); B) A plus superoxide dismutase (30 μg); C) A plus catalase (30 μg); D) A plus catalase (30 μg) and superoxide dismutase (30 μg). Total reduction of cytochrome c by dithionite in this system is indicated by the dashed line. Curves shown are tracings of actual curves recorded with a Beckman Model 25 recording spectrophotometer (Beckman Instruments, Inc., Irvine, Calif.)

Figure 2: Kinetics of $CrO_4^=$ leak from liposomes: o----o, hypoxanthine (0.14 μmol); ●——●, hypoxanthine plus xanthine oxidase (0.013 U); ▲——▲, hypoxanthine plus xanthine oxidase plus superoxide dismutase (40 μg). The curve shown for hypoxanthine alone was identical to that observed when liposomes were suspended in buffered saline.

leased minimal amounts of previously sequestered $CrO_4^=$. The rate of release of $CrO_4^=$ was not enhanced by the addition to the reaction mixtures of either xanthine oxidase or hypoxanthine, but was increased significantly if both enzyme and substrate were added simultaneously. The kinetics of $CrO_4^=$ release in a representative experiment are shown in Figure 2. The enhanced $CrO_4^=$ release from liposomes exposed to the complete $O_2^-\cdot$ generating system could be inhibited significantly by either superoxide dismutase or catalase (Table I). Neither of these enzymes influenced release of $CrO_4^=$ from liposomes suspended in buffered saline. The greatest inhibition of $CrO_4^=$ release from liposomes exposed to the complete $O_2^-\cdot$ generating system occurred when superoxide dismutase and catalase were added simultaneously. Super-

TABLE I

$CrO_4^=$ LEAK FROM LIPOSOMES

Liposomes exposed to:	(n)	% $CrO_4^=$ Leak*
Buffered saline	(6)	10.9 ± 1.5
Hypoxanthine (0.14 μmol)	(15)	11.5 ± 1.7
Xanthine oxidase (0.013 U)	(6)	13.0 ± 2.1
Hypoxanthine + xanthine oxidase	(15)	38.5 ± 3.6‖
+ superoxide dismutase (40 μg)	(15)	21.6 ± 2.5 (-63%)§
+ catalase (10 μg)	(6)	26.4 ± 5.3 (-45%)¶
+ superoxide dismutase + catalase	(7)	19.3 ± 2.3 (-72%)§
+ superoxide dismutase (boiled)	(3)	36.2 ± 5.8
+ bovine serum albumin (40 μg)	(3)	37.1 ± 6.5

*Expressed as percent of total $CrO_4^=$ released from liposomes by 0.2% Triton X-100 at 24 hours. Mean ± S.E.M.
‖p vs. hypoxanthine alone ≤ 0.001 (Student's t test).
§Percent inhibition of $CrO_4^=$ release (minus hypoxanthine alone) $p < 0.001$
¶$p < 0.025$

oxide dismutase, which had been boiled for 30 minutes (with a consequent loss of 65% of its activity), was incapable of reducing $CrO_4^=$ release. Similarly, at identical concentrations, nonenzymatic proteins such as bovine serum albumin were without any effects in this system. Appropriate control experiments excluded the possibility that any of the reagents influenced the measurement of $CrO_4^=$.

DISCUSSION

A role for oxygen-derived free radicals in the production of membrane injury has now been demonstrated in a variety of experimental systems (4-9, 15, 16). Both the precise nature of the reactive molecules which mediate such injury and the mechanism whereby integrity of lipid bilayers is lost are still controversial. Results of most studies, however, can be interpreted as indicating roles for both $O_2^-\cdot$ and H_2O_2 or,

more specifically, products of their interaction such as OH· and singlet oxygen (6, 7, 9, 15, 16). That these reactive species can produce lipid peroxides has been surmised and, in some instances, demonstrated (6-8, 17); but whether lipid peroxidation per se can account for alterations in membrane integrity is not entirely clear.

The results of the experiments described in this report can be interpreted as indicating a role for $O_2^-\cdot$ plus H_2O_2 in the perturbation of artificial lipid bilayers. Both $O_2^-\cdot$ and H_2O_2 are generated by the action of xanthine oxidase on hypoxanthine and both superoxide dismutase and catalase were capable of reducing leakage of $CrO_4^=$ from liposomes suspended in this system. Failure of these enzymes to reduce $CrO_4^=$ release to control levels was not unexpected since a wide variety of secondary interactions between the enzymes and their products have been observed previously. For example, H_2O_2 is capable of inactivating superoxide dismutase (18) while at the same time generating a product which, by itself, is capable of promoting peroxidation of lipids (19). Thus, the precise nature of the products which mediate the effects upon liposomes which we observed in our studies cannot be adduced from the data obtained. Nevertheless, it is clear that such products generated by the action of xanthine oxidase on hypoxanthine, and presumably related to $O_2^-\cdot$, were capable of perturbing the lipid bilayers of multilamellar liposomes sufficiently to cause leakage of relatively impermeant anions from these artificial membrane structures.

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