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RADIOPROTECTIVE EFFECT OF SUPEROXIDE DISMUTASE ON MODEL PHOSPHOLIPID MEMBRANES

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SUMMARY

1. Hydroperoxide formation in model membranes was measured via the net increase in absorbance at 232 nm after exposure to X-rays or ^{137}Cs gamma rays in the presence and absence of bovine superoxide dismutase and other radical scavengers.

2. Membranes X-irradiated in air to 4200 rad at 210 rad/min exhibited a large increase in absorbance, a major portion of which was O_2^--mediated since active superoxide dismutase at 1 μg/ml reduced it by more than 80 % to the level observed in N_2O. In N_2 the change in absorbance was smaller than in N_2O but not in proportion to the halving in OH production.

3. The net absorbance of membranes exposed to a constant dose from ^{137}Cs increased with decreasing dose rate. A minor component of this effect was due to exposure protraction with decreasing dose rates while the major component was attributed to long chain reactions initiated by ionizing radiation. A corollary effect was also observed, namely, that with reducing dose rate the dose required to elicit a constant absorbance change decreased. Both aspects were abolished by superoxide dismutase at 1 μg/ml.

4. The enzyme protected membranes after an acute exposure and from low level radiation at natural background while its inactivated form sensitized.

INTRODUCTION

Many biochemical and physiological processes in living organisms are regulated by membranes whose structure is currently viewed as a mosaic array of protein particles floating in a double-layered sheet of lipid molecules [1]. The response of membrane systems of this type to ionizing radiation may therefore be governed both by the protein and lipid moieties. For instance, radiation-induced changes in membrane permeability have been studied extensively and are related to damage of the protein components [2–7]. Recently, it was also shown that the rate of loss of the proliferative capacity of cells by ionizing radiation varied inversely with the sulfhydryl content of the cell membrane [8].

Radiation-induced damage to the lipid moiety is unusual in that, at the structural level, a protracted radiation dose is more effective than a shorter, more intense one of larger size [9]. In principle, this dose rate effect is accountable in terms of slowly-progressing long chain reactions, initiated by ionizing radiation but sustained without it. At a constant dose, the radiation-induced chemical effect should increase with decreasing dose rate. As this paper will show, the phenomenon is observed with model phospholipid membranes whose polar regions possess an interfacial potential capable of perturbing the radiation chemistry in the adjacent aqueous phase through efficient trapping of charged species produced during water radiolysis [10]. The superoxide anion (O_2^-) and the hydrated electron (e_{aq}^-) are particularly subject to this electrochemical trapping mechanism at membrane surfaces [10, 11]. However, with aeration, the e_{aq}^- reacts rapidly with oxygen (reaction 1 [12]).

$$O_2 + e_{aq}^- \xrightarrow{k_1 = 1.88 \cdot 10^{10}\,M^{-1}\,sec^{-1}} O_2^- \tag{1}$$

so that the superoxide anions are the dominant species collected.

The interaction of O_2^- with membranes is varied. It may participate in redox reactions with suitable cations sorbed to the membrane surface [10] or oxidize sulfhydryl residues in membrane proteins [8, 13]. On the other hand, the chemical dismutation of O_2^- (reaction 2 [14–16]) is reported to result in singlet oxygen ($^1O_2$),

$$2O_2^- + 2H^+ \xrightarrow{k_2 \simeq 100\,M^{-1}\,sec^{-1}} {}^1O_2 + H_2O \tag{2}$$

a much more efficient initiator of hydroperoxide (ROOH) formation in the unsaturated fatty acids (RH) of membranes than ground state oxygen [17, 18]. The generation of $^1O_2$ from superoxide is inhibited by the enzymatic dismutation of O^-_2 with superoxide dismutase according to reaction 3 [15, 16, 19, 20] to

$$O_2^- + O_2^- + 2H^+ \xrightarrow[\text{superoxide dismutase}]{k_3 = 2.3 \cdot 10^9\,M^{-1}\,sec^{-1}} O_2 + H_2O_2 \tag{3}$$

ground state oxygen and H_2O_2. This enzyme may therefore be expected to protect membranes from oxidative damage by either O^-_2 or $^1O_2$ and recently, its protective action against biochemical peroxidation has been demonstrated in a number of membrane systems [21–24]. In the work reported here, the radioprotective effect of superoxide dismutase on model phospholipid membranes is examined and observed to apply over a wide range in dose rate, extending from natural background to several hundred rad/min.

MATERIALS AND METHODS

Chemicals. Lipids from both soybeans and fresh beef brain were used. The extraction, purification, and characterization of the latter has been described previously [25]. The soybean lipid fraction was obtained from selected samples of Azolectin (Associated Concentrates, Woodside, Long Island, N.Y.). By weight, the dry granules contained Mg, Ca, Ga, Si, Al, Fe, Cu, Mn, Ti, and K in 4557, 2418, 93, 47, 28, 8370, 7.4, 7, 20, and 15 810 ppm, respectively. In the membrane preparations these impurities were diluted 1000-fold or more, thus reducing still further any component of peroxidation due to metal catalysis [26]. The sulphur content was less than

1000 ppm by weight. By thin-layer chromatography [25] the major components were found to be phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine, while sterols, phosphatidic acid, and phosphoinositides were present in smaller amounts. The major fatty acids present were linoleic acid (58 %), oleic acid (10 %) and palmitic acid (24 %). All other chemicals were reagent grade. The N_2O and N_2 were obtained from Matheson of Canada Limited, Whitby, Ontario. The former was research grade and contained < 10, 5, 2, and 5 ppm of CO_2, O_2, NO_2, and NO, respectively. The latter was of 99.9 % purity.

Superoxide dismutase. Bovine erythrocyte superoxide dismutase was obtained as a lyophilized powder (Biochemical Division, Truett Laboratories, Dallas, Texas) and had a specific activity of 3000^+ units/mg. The enzyme dismutates superoxide at a rate of $2.3 \cdot 10^9\ M^{-1}\ sec^{-9}$ [15].

Membrane preparation. The soybean lipid fraction was first sterilized by dissolution in chloroform for 10 min and then dried under vacuum. All subsequent operations were aseptic using steam sterilized equipment. A sample (1 g) was homogenized in 30 ml of distilled, deionized water for 5 min in a motor driven Potter-Elvehjem tissue homogenizer. The homogenate was sonicated at 0 °C for 60 min in a bath-type sonicator and then centrifuged at $110\,000 \times g$ for 30 min. The concentration of lipid in the supernatant containing the membrane particles was adjusted to the desired value by dilution. On freeze-fracture and etching electron microscopy the membrane particles were spherically shaped with an average diameter of 21 ± 5 nm [27]. All membrane samples were irradiated in the dark and in distilled water to minimize photochemical and metal-catalyzed autoxidation reactions [26], as well as the possible conversion of O_2^- and H_2O_2 to hydroxyl (OH) radicals by the Haber-Weiss reaction [28]. The pH of the samples was in the 6.5–7.0 range.

Irradiation. For short term, high dose rate (210 rad/min) exposures at ambient temperature, a Westinghouse 250 KV therapy X-ray unit was used at 200 KV and 15 mA. The X-ray tube had an inherent filtration equivalent to 2 mm Al plus an additional filter of 1 mm Al. The samples were continuously gassed by bubbling with the desired gas before, during, and after the irradiation for the duration of the experiments.

For longer term, low dose rate (100 rad/min or less) exposures, a twin source ^{137}Cs irradiator (A.E.C.L. Gammabeam), containing 2 and 100 Ci sources, was used. With it, dose rates of 0.6 mrad/min and higher were available depending on the source strength and the source-target distance. The dosimetry was done with LiF discs (courtesy Dr. H. M. Johnson). Control of the temperature of the samples was achieved by means of a constant-temperature plate connected to a re-circulating water bath. All irradiations were in air without bubbling during the exposures.

Absorbance measurement. The radiation-induced hydroperoxide formation was assessed in a dual beam spectrophotometer by measuring the absorption in the 200–300 nm range and noting the net difference in absorbance (ΔA) at 232 nm of exposed and unexposed membrane samples. In general, no changes in absorbance were observed at 290–300 nm. These measurements were made immediately after an irradiation and, in some instances, at specified times thereafter in aliquots which were subsequently discarded.

RESULTS

The dominant mono-hydroperoxides formed in unsaturated fatty acids, exposed to radiation, are conjugated dienes that absorb at 232 nm [29]. The extent of dienoic conjugation, as determined by absorbance measurements, has been shown to parallel alternative methods of analysis [29] and is dependent on the lipid concentration [30]. This dependence on the critical micelle concentration is illustrated in Fig. 1 which also shows that the net increase in absorbance of irradiated membranes is greater after sonication. Examination of both sonicated and unsonicated membranes by electronmicroscopy showed that the latter were predominantly multilayered micelles whereas the former, spherically-shaped particles consisted mostly of bilayers as previously described [31]. On a unit weight basis the sonicated membranes must, therefore, have a larger surface area directly in contact with the water to more efficiently trap the superoxide anions generated by ionizing radiation. They were consequently expected to show a greater radiation response as was observed (Fig. 1). Qualitatively similar results were obtained with membranes prepared from the phospholipids of fresh beef brain.

It will be noted from Fig. 1 that approx. 550 μg/ml is the critical micelle concentration at which optimal hydroperoxide formation occurred during the irradiation of sonicated membranes. However, it is known that the original hydroperoxides generate additional conjugated hydroperoxides by a relatively slow free-radical chain reaction [17, 18, 30]. The accumulation of these secondary conjugated products was observed (Fig. 2) in samples where the amount of lipid present (150 μg/ml) was well below the

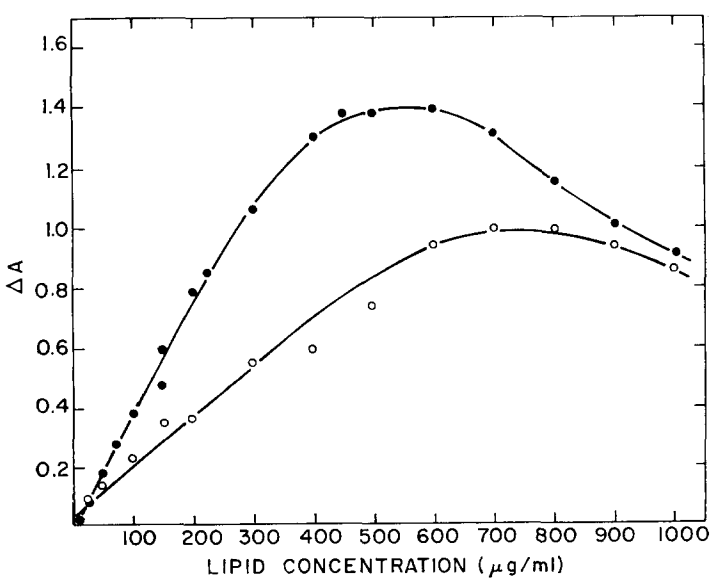

Fig. 1. Variation in the differential absorbance, ΔA (Irradiated-unirradiated) at 232 nm with lipid (soybean) concentration of sonicated (●—●) and non-sonicated (○—○) membranes. Absorbance measurements were made immediately after the irradiated membranes had been exposed to 4200 rads of 200 kV, 15 mA X-rays at 210 rad/min. Temperature 25 °C.

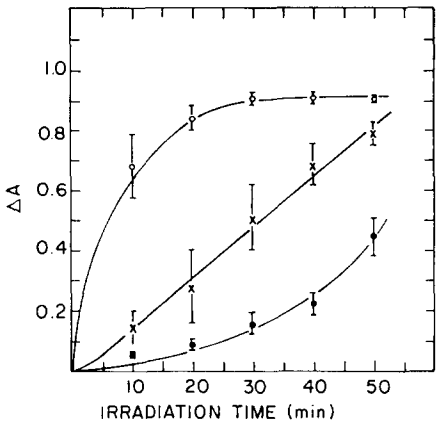

Fig. 2. Increase with irradiation time in the differential absorbance ΔA of irradiated minus unirradiated model lipid (soybean, 150 μg/ml) bilayer membranes as measured immediately after the X-irradiation (● — ●), 90 min (× — ×), and 270 min (○ — ○) later. Irradiation in air with 200 kV, 15 mA X-rays at 210 rad/min. Temperature 19 °C. Error bars represent standard deviation. Increase in unirradiated membrane samples after 270 min $\leqq$ 0.01 absorbance unit.

critical micelle concentration, thereby reducing the amount of hydroperoxides formed during the irradiation and extending a larger portion of the overall process into the post-exposure period. Thus, whereas the amount of hydroperoxides formed after a 10 min irradiation (at 210 rad/min and 19 °C) was equivalent to 0.05 of an absorbance unit, it increased by a factor of approx. 13.6 to 0.68 in the following 270 min (Fig. 2). During this same period the hydroperoxide level reached a maximum in samples irradiated for 30 min or longer and reflects both the increase in initiation of hydroperoxide formation with dose as well as the growing contribution by the free-radical mechanism [26]. The latter process accounts for the non-linear increase in ΔA with irradiation time, as measured immediately after the radiation exposure (Fig. 2).

The chain reaction by which autoxidation of unsaturated fatty acids in phospholipids is propagated, has been investigated over many years [18, 30, 32]. The proposed free-radical mechanism does not include a role for some of the products of water radiolysis which, in addition to the e_{aq}^- referred to before, also elaborates hydroxyl radicals (OH), hydrogen atoms (H), hydrogen peroxide (H_2O_2) and hydrogen (H_2) according to reaction 4, where

$$H_2O \xrightarrow{h\nu} OH\ (2.74),\ e_{aq}^-\ (2.76),\ H\ (0.55),\ H_2O_2\ (0.72),\ H_2\ (0.55) \qquad (4)$$

the numerical values in parentheses refer to the yield of the different species per 100 eV of absorbed energy (33). The involvement of OH and H_2O_2 in biochemical oxidative damage of membranes has been suggested and disputed [21, 23, 24, 34]. Irradiation studies under a variety of radical scavenging conditions allow an assessment to be made of the relative magnitude of membrane damage as mediated by the different reactants. Thus, in N_2O, the e_{aq}^- is converted to OH, doubling its yield [35] and if the OH radicals are involved in radiation-induced hydroperoxide formation, membranes irradiated in N_2O would show increased absorbance at 232 nm. The results in Figs. 3 and 4 illustrate that for model bilayer membranes, prepared from soybean and brain

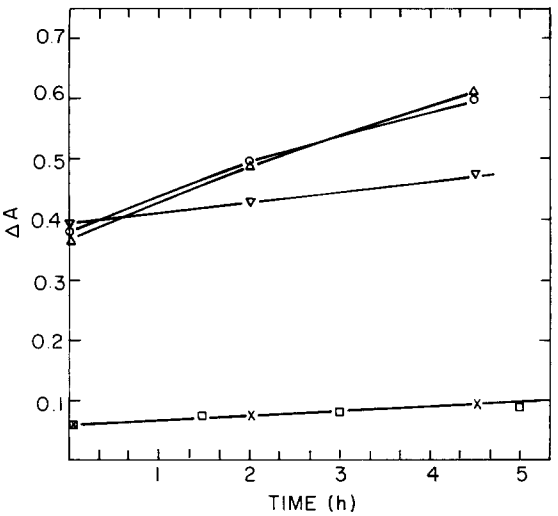

Fig. 3. Variation with post-irradiation time in the differential absorbance ΔA of irradiated minus unirradiated model lipid (soybean, 150 μg/ml) bilayer membranes in air without additives (△—△), N_2O without additives (□—□), air plus 1 μg/ml of superoxide dismutase, added before the irradiation (×—×), air plus 1 μg/ml of inactivated superoxide dismutase, adddd before the irradiation (○—○), and air plus 1 μg/ml of superoxide dismutase, added 5 min after the irradiation (▽—▽). Irradiation in water with 200 kV, 15 mA X-rays at 210 rad/min for 20 min. Temperature 24 °C.

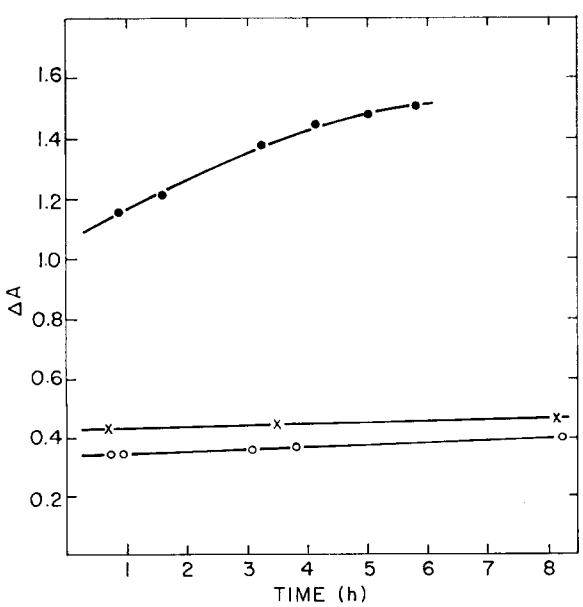

Fig. 4. Variation with post-irradiation time in the differential absorbance ΔA of irradiated minus unirradiated model lipid (beef brain 365 μg/ml) bilayer membranes in air (●—●), N_2O (×—×), and N_2 (○—○). Irradiation in water with 200 kV, 15 mA X-rays at 210 rad/min for 30 min. Temperature 24 °C.

phospholipids, respectively, and irradiated in N_2O at a dose rate of 210 rad/min, the major change in absorbance occurred during the exposure, consistent with an OH-mediated attack on the fatty acid constituents. The relatively minor increase in absorbance over an extended time in the post-irradiation period cannot be due to the short-lived OH, and may be due to the much longer lived H_2O_2 or to the oxygen impurity in the N_2O and was not investigated further. Relative to its level in N_2O, hydroperoxide formation was only reduced by about 20 % in membranes irradiated in N_2 (Fig. 4) in which, as already noted, OH production is halved. This quantitative discrepancy suggests that e^-_{aq} may react with unsaturated fatty acids to form hydroperoxides after being scavenged from the aqueous phase by the electric potential of the membrane surfaces. The reduction in layer spacings in electron-bombarded lipid bilayers has been attributed to radiation damage of the fatty acids [36] and it seems likely that the hydrated electron would also react with these membrane constituents provided they are accessible to it.

In air, hydroperoxide formation is enhanced by a factor of approx. 3 or more as shown by the results in both Fig. 3 and fig. 4, where the net change in absorbance of exposed membranes was measured shortly after the irradiation and at various times thereafter. Superoxide dismutase, at a concentration of 1 μg/ml, prevented the increase in hydroperoxide formation by reducing it to the level observed in N_2O (Fig. 3) while the inactivated enzyme had no effect. Therefore, in view of the specificity with which the enzyme dismutates O_2^- to H_2O_2, these results clearly implicate O_2^- in the formation of conjugated hydroperoxides, either directly or through its $^1O_2$ derivative. The direct involvement of O_2^- has not been established chemically and requires a detailed study that is beyond the scope of this paper. On the other hand, $^1O_2$ is known to be a rapid initiator of hydroperoxide formation and could, in principle, account for the present data. The fact that the inactivated enzyme had no radioprotective effect, despite its apparent ability to scavenge $^1O_2$ non-catalytically [16, 37–40], does not constitute contrary evidence since, at 1 μg/ml, the concentration was at least three orders of magnitude too low for the inactive enzyme to be effective. While the yield of $^1O_2$ from the chemical dismutation of O_2^- is at present uncertain, it must be kept in mind that the collection and probable concentration of superoxide anions within the electric field of the membrane surfaces [10] may well catalyze the reaction. As will be discussed later, the involvement of either O^-_2 or $^1O_2$ in initiating hydroperoxide formation may account for the ability of superoxide dismutase to protect irradiated membranes in the post-irradiation period (Fig. 3).

Radiation-induced hydroperoxide formation in membranes increases with decreasing dose rate by virtue of its time dependence and the free radical nature of the chain propagating reactions. The supporting data are presented in Fig. 5 which show the variation in levels of conjugated hydroperoxides in unprotected model lipid (soybean) membranes irradiated to graded doses at variable dose rates. The family of curves shows that the chemical effect from a constant dose increases with decreasing dose rate over a 60-fold difference in dose. Qualitatively similar results were obtained with membranes composed of beef brain phospholipids. A minor component of this dose rate effect is due to protraction of the exposure period necessitated by the procedure of irradiating the membranes to constant dose at decreasing dose rates. For example, the very slight net change in absorbance (< 0.02 unit), exhibited by membranes immediately after being irradiated to 600 rads at 100 rad/min (Fig. 5), in-

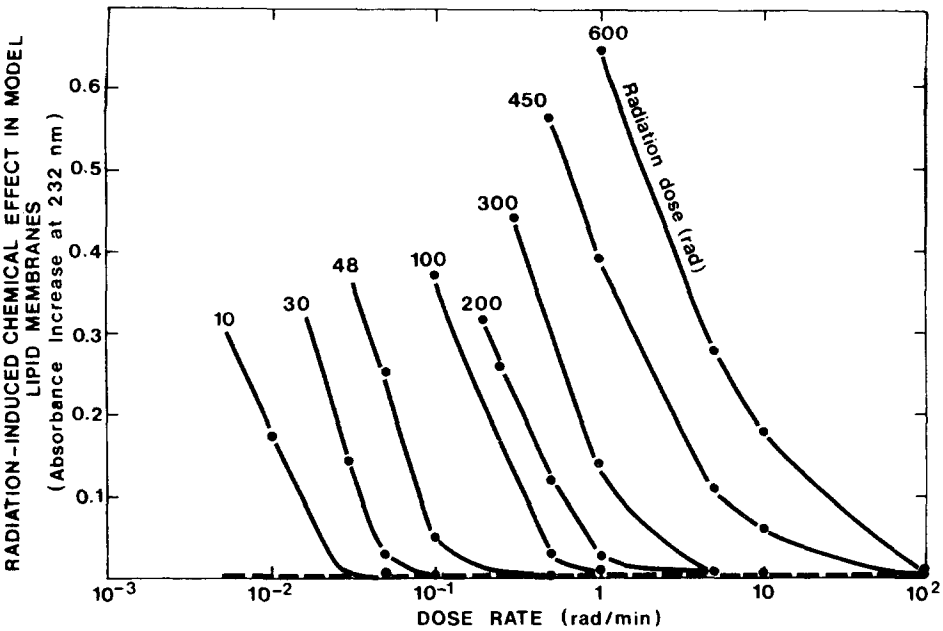

Fig. 5. Variation in level of conjugated hydroperoxides, absorbing at 232 nm, in model lipid (soybean, 150 μg/ml) membranes irradiated at variable dose rates and to different doses with ^{137}Cs gamma rays in air and in the presence (– – –) and absence (●—●) of superoxide dismutase (1 μg/ml). Qualitatively similar results were obtained with membranes composed of beef brain phospholipids. The change in the differential absorbance of irradiated minus unirradiated membranes was measured immediately after the exposure. Temperature 25 °C.

creased after 10 and 16.7 h to 0.17 and 0.30, respectively. The former value is only approx. 25 % of the increase in absorbance observed with membranes irradiated for 10 h to 600 rad at 1 rad/min and this percentage was observed to decrease at the intermediate dose rates where the required exposures became shorter. It is therefore concluded that protraction of the exposure period is a minor cause of the dose rate effect illustrated in Fig. 5. The major component is effected by continuous irradiation, consistent with radiation initiating the formation of hydroperoxides which then propagate by the free radical mechanism [17, 18, 26]. In nominally unirradiated membranes, hydroperoxide formation at 25 °C was minor except for the longest exposure of 16.7 h (1000 min), where its extent was rather variable (Fig. 6).

The set of curves in Fig. 5 also indicates that with reducing dose rate the dose required to elicit a constant chemical effect decreases. This point is clearly illustrated in Fig. 7 for $\Delta A = 0.3$. The result is analogous to the dose-rate dependence of the dose required to break the structure of model phospholipid membranes by ^{22}Na emissions [9]. This dependence on dose rate of the chemical effect is completely wiped out by the radioprotective action of superoxide dismutase as shown by the horizontal broken line adjacent to the abscissa in Fig. 5. No difference greater than 0.01 absorbance unit was found between exposed and unexposed membranes in the presence of the enzyme at 1 μg/ml. Within the 0.01–10 rad/min range in dose rate, therefore, the superoxide-mediated reactions dominate radiation-induced hydroperoxide formation in the mem-

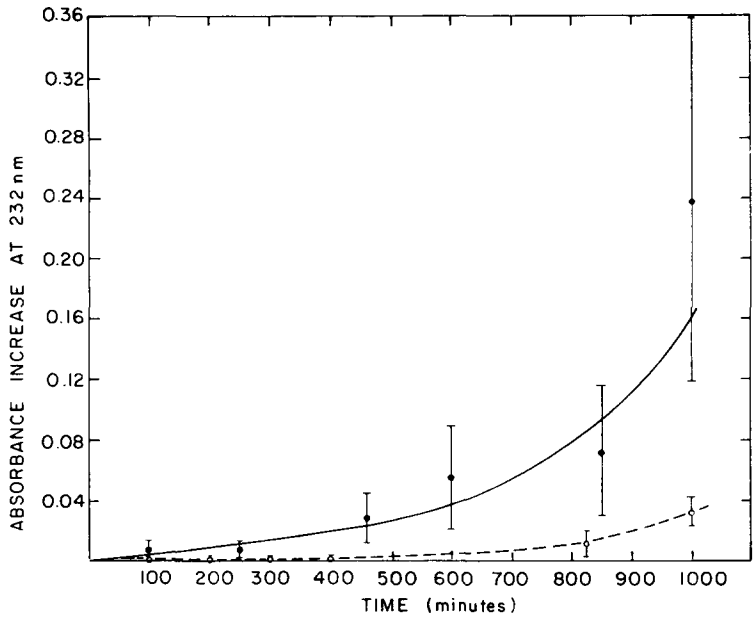

Fig. 6. Increase with time in absorbance at 232 nm of control, unexposed membranes in the presence (○—○) and absence (●—●) of superoxide dismutase (1 μg/ml). Error bars represent standard deviation. Temperature 25 °C.

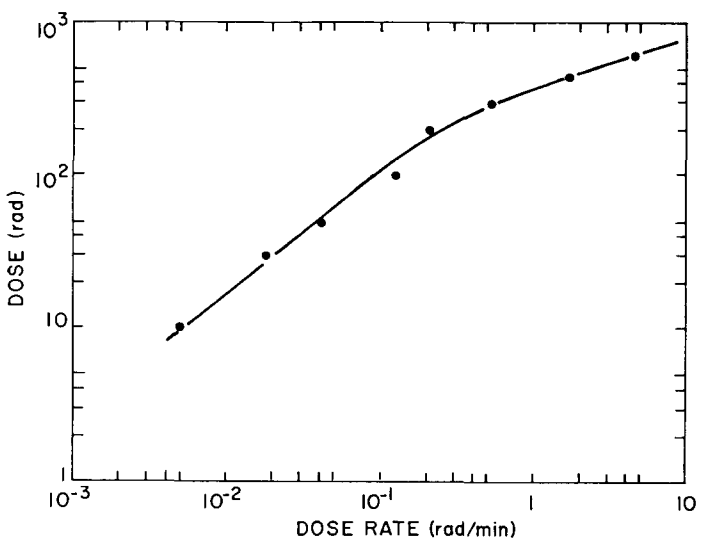

Fig. 7. Dose rate dependence of the dose required to produce a constant chemical effect ($\Delta A = 0.3$) in model lipid (soybean, 150 μg/ml) membranes irradiated with ^{137}Cs gamma rays. The graph was derived from Fig. 5 by extending a horizontal line through the family of curves at 0.3 absorbance unit and projecting its points of intersection at the 10–600 rad dose lines to the dose rate axis to obtain the required dose rate values.

branes and minimizes any involvement of H_2O_2 or OH. The latter finding contrasts with the result obtained at 210 rad/min where it was found (Fig. 3) that the enzyme only reduced the hydroperoxides to the level observed in membranes irradiated in N_2O. It therefore appears that OH-mediated oxidation of unsaturated fatty acids in membranes is negligible with doses of 600 rad or smaller at 100 rad/min or less and only becomes significant at higher dose rates and doses where, concomitantly, O_2^- involvement fades.

Straightline extension of the graph in Fig. 7 to lower dose rates suggested that the membranes might respond within approximately 167 h in a measurable way ($\Delta A = 0.3$) to ionizing radiation energy of natural background intensity. If so, this would account for the observation that, in the presence of superoxide dismutase, the so-called unexposed membranes exhibited less autoxidation than when without it (Fig. 6) and, at the same time, provide an explanation for the radioprotective effect of the enzyme in the post-irradiation period (Fig. 3). Accordingly, membranes with and without superoxide dismutase (at 1 μg/ml) were stored under otherwise identical conditions at two locations characterized by radiation fields of $2.6 \cdot 10^{-6}$ and $0.75 \cdot 10^{-6}$ rad/min, respectively. The time-dependent changes in absorbance at 232 nm in the different membranes occurred in much less than 167 h, and are given in Fig. 8. It shows that, at natural laboratory background ($0.75 \cdot 10^{-6}$ rad/min), the level of hydroperoxides was generally less than at $2.6 \cdot 10^{-6}$ rad/min where it reached the saturation point within 28 h. Comparing the two curves, it is clear that the reduction in the chemical effect at $0.75 \cdot 10^{-6}$ rad/min is less than the 3.5-fold reduction in dose rate, thus suggesting that even at very low radiation intensities the membranes contin-

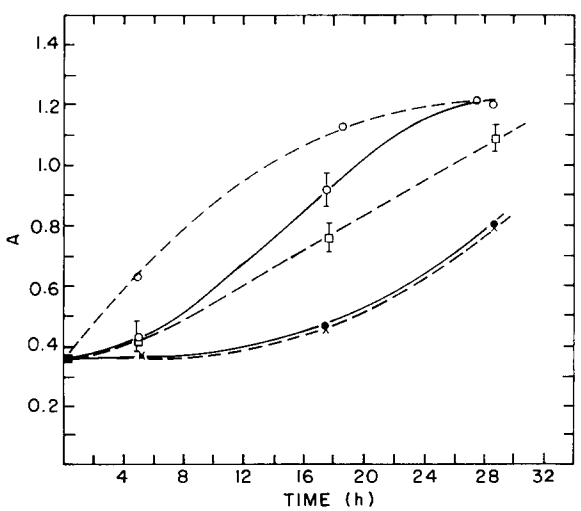

Fig. 8. Change with time in absorbance at 232 nm of lipid membranes (soybean, 150 μg/ml), exposed to $2.6 \cdot 10^{-6}$ rad/min in the absence (○—○) and presence (●—●) of 1 μg/ml of superoxide dismutase, as well as of membranes of the same type exposed to $0.75 \cdot 10^{-6}$ rad/min in the presence (×—×) and absence (□—□) of the enzyme. The change in absorbance of membranes exposed to $2.6 \cdot 10^{-6}$ rad/min in the presence (○—○) of inactivated superoxide dismutase (1 μg/ml) is also shown. Dose rates based on LiF dosimetry (courtesy Dr. H. M. Johnson). Standard deviations given by error bars unless smaller than symbol size. Temperature 25 °C.

ue to reflect the type of dose rate dependence illustrated in Fig. 7. Further, for either dose rate, the radioprotective effect of superoxide dismutase is shown both by the delay in onset of hydroperoxide formation and by its subsequent control to a level that was invariant with dose rate but increased with time. The latter phenomenon may represent facile autoxidation of unsaturated fatty acids by ground state oxygen as previously understood [17].

Fig. 8 also shows the enhancement of lipid peroxidation at near natural background by inactivated superoxide dismutase. This sensitization effect is not abolished after dialysis of the inactivated enzyme against doubly distilled water. Free Cu^{2+} is therefore an unlikely cause of the effect. More probably, the large molecules of the inactivated enzyme enter the hydrocarbon region of the membranes to react with the fatty acids. Inside the membranes the denatured enzyme may be more reactive through activation of its residual sulphydryl groups and contribute to the initiation of hydroperoxide formation [41]. As well, a lipidprotein matrix is known to trap free radicals which gradually degrade the lipid moiety by peroxidation [42].

DISCUSSION

A role for $^1O_2$ in the initiation of hydroperoxide formation in lipids during photosensitization reactions has previously been proposed to overcome both energy and spin barriers [17]. Its relevance to lipid membranes stored in darkness under natural laboratory conditions may be questioned since no source of $^1O_2$ is identifiable from the scheme. This difficulty may be removed, however, if the radioprotective effect of superoxide dismutase on so-called unexposed membranes, observed in the present study (Fig. 6), is interpreted to signify that a forward component of facile autoxidation of unsaturated fatty acids in the phospholipids of membranes is radiation-induced. Unless catalytically removed by superoxide dismutase [20], the superoxide anions, generated in the aqueous phase, may initiate hydroperoxide formation directly, or indirectly by chemical dismutation to $^1O_2$ [14–16]. In accordance with this account, lipid peroxidation in the membranes should vary with the radiation intensity at and near natural background, be retarded by superoxide dismutase, and reduced by the enzyme to a common level, irrespective of the intensity of the radiation field. The data in Figs. 6 and 8 illustrate that all three criteria were fulfilled.

The emergence of superoxide dismutase as an effective protector against peroxidation of model membranes is of further interest in that it provides a framework for expecting variable protection of cellular membranes, depending on whether the enzyme is associated with them and to what extent. Inadequate protection would render the membranes susceptible to both radiation and biological damage from O^-_2 toxicity.

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