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THE EFFECTS OF FREE RADICALS AND OXYGEN ON THE ACTIVITY OF
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND THE MEMBRANE
PERMEABILITY OF RESEALED ERYTHROCYTE GHOSTS

by

Spencer Kam-lun Kong
B.Sc., Hong Kong University, 1973

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Kinesiology

Spencer Kam-lun Kong 1979
SIMON FRASER UNIVERSITY
July 1979

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Title of Thesis/Project/Extended Essay

The effects of free radicals and oxygen on the activity of glyceraldehyde-3-phosphate dehydrogenase and the membrane-permeability of revealed erythrocyte ghosts

Author:

Kam-Fung Kong

Date: 15 August 1979
Title of Thesis: The effects of free radicals and oxygen on the activity of glyceraldehyde-3-phosphate dehydrogenase and the membrane permeability of resealed erythrocyte ghosts.

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ABSTRACT

Free radical forms of oxygen are known to cause damage to living cells and tissues, and oxygen plays an ambivalent role in both enhancing or retarding cellular free radical processes. A study of free-radical damage to plasma membranes was undertaken to obtain a better understanding of the mechanisms of free-radical damage to living cells involving (1) the relative effectiveness of \( \text{H}_2\text{O}_2 \), \( \cdot\text{OH} \), \( \text{O}_2^- \) and \( \cdot\text{e}^- \); (2) interactions between these radicals; and (3) the effects of oxygen.

Membranes were isolated from human erythrocytes in the form of resealed ghosts which are impermeable to polar, low molecular weight non-electrolytes. Damage was estimated from (1) the permeability of membranes to glyceraldehyde-3-phosphate (G3P) and NAD, and (2) the inactivation of membrane-bound G3P dehydrogenase (G3PDH). The radiolysis of water by gamma-irradiation was used as the source of free radicals: \( \text{H}_2\text{O}_2 \), \( \cdot\text{OH} \), \( \text{O}_2^- \) and \( \cdot\text{e}^- \).

Ghosts were more susceptible to irradiation than erythrocytes by an order of magnitude.
Using appropriate mixtures of scavengers such as catalase, superoxide dismutase and formate, the rates of increase in permeability ($R(\text{mb})$) and loss of activity of G3PDH ($R(\text{enz})$) per molecule of each kind of free radicals were measured. $R(\text{enz})$ of O2- and H2O2 were 12-fold, and of .OH 17-fold that of e-. Hence with respect to $R(\text{enz})$: .OH > O2- > H2O2 > e-. $R(\text{mb})$ of e- and H2O2 were about 1.5-fold and of .OH 3-fold that of O2-. Hence, with respect to $R(\text{mb})$: .OH > H2O2 = e- > O2-. Comparison between H2O2 added as a chemical reagent and H2O2 formed by irradiation showed that membranes or G3PDH were relatively inert to reagent H2O2 but markedly susceptible to the latter.

The interactions between free radicals and oxygen were studied, and the following mixtures were found to induce greater damage than that which could be accounted for by the independent actions of the constituent free radicals: (1) .OH + H2O2, and (2) .OH + H2O2 + O2-. In contrast, the following mixtures were found to induce less damage: (1) H2O2 + O2-, and (2) oxidizing radicals (.OH, H2O2) + reducing radicals (e-, H.). The results suggest that a Haber-Weiss like interaction occurs between H2O2 and O2- and
also between H2O2 and .OH to produce a species more potent than either in causing increased permeability. The decrease in damage observed in the simultaneous presence of oxidizing and reducing radicals reveals an antagonistic effect by which each tends to moderate the damage by the other.

Permeability was 10-fold more resistant to free-radical damage than G3PDH in air-saturated media. However, further increases in oxygen concentrations from 230 μM to 1150 μM in media containing (i) no scavengers, (ii) formate, (iii) catalase plus dismutase, increased the aerobic rates of increase in permeability by 60-, 7.5- and 9.5-fold respectively, but increased the aerobic rates of enzymic inactivation by 2.7-, 4.2- and 5.5-fold. Though enzymic inactivation is more sensitive than permeability to the presence of small amount of oxygen (less than 230 μM) the latter is more sensitive at higher concentrations of oxygen (above 230 μM). These differences in sensitivity to oxygen are independent of the nature of free radicals causing the damage. A mechanism is therefore proposed for the "oxygen effect" in radiation damage in which O2 (1) interacts with e- and H., producing O2-, thus allowing a Haber-Weiss like reaction with H2O2, and (2) the further increases in damage.
at increased concentrations of oxygen are primarily due to interactions of oxygen with sites of initial damage.

The order of effectiveness of free radical scavengers in inhibiting the increase in permeability caused by gamma-irradiation was:

formate (90%) > nitrogen (65%) > catalase (60%) > dismutase (32%)

and with respect to enzymic inactivation,

nitrogen (100%) > formate (77%) > dismutase (48%) = catalase (44%)

Evidently, free-radical damage to tissues is only partly due to the direct actions of individual free radicals, a substantial fraction being both the result of interactions between the radicals and of the propagation of initial damage in free radical chain reactions, with oxygen amplifying the chain propagation by increasing branching.
to Allan.
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GENERAL INTRODUCTION

All respiring organisms are caught in a cruel conflict in that the oxygen which supports their existence is a toxic substance in whose presence they survive only by virtue of an elaborate system of defences (Pridovich 1975). The toxic effects of oxygen are caused by the intermediates formed in the univalent pathways of reduction to water. Intermediates such as O$_2^-$, ·OH, and H$_2$O$_2$ are formed as electrons are added one by one as shown in the following scheme:

\[
\begin{align*}
O_2 + e^- \rightarrow & \rightarrow O_2^- \\
O_2^- + e^- + 2H^+ \rightarrow & \rightarrow H_2O_2 \\
H_2O_2 + e^- \rightarrow & \rightarrow ·OH + OH^- \\
·OH + e^- + H^+ \rightarrow & \rightarrow H_2O
\end{align*}
\]

These intermediates are free radicals, which are chemical species having a lone unpaired electron in an outer orbital. The strong tendency to pair this lone electron makes free radicals very reactive. Undoubtedly, the accumulation of
such species in a living cell would result in damage to the molecular fabric of the cell. In fact, these radicals are continually produced in low concentrations during cellular metabolism. Substantial quantities of catalase, superoxide dismutase, glutathione peroxidase, and sulphhydryl compounds in cells of aerobic organisms have evolved to provide adequate protection under ordinary conditions, but pathological situations arise in which such defences are overpowered, for instance, in irradiation or chemotherapy. Evidence of toxicity of these free radicals in biological systems is abundant: glucose-6-phosphate dehydrogenase deficient haemolytic anaemia, erythropicetic protoporphyria, carcinogenesis, aging, radiation sickness, and brain and lens damage by pro-oxidant drugs etc. However, little is known of the ways by which the radicals produce their toxic effects. Since the functional integrity of the plasma membrane of a cell is vital for its existence, and since the unsaturated fatty acids of membranes are known to be susceptible to damage by free radicals, the membrane constitutes one of the important sites in the cell of which damage by free radicals may explain the toxic effects of these radicals. Therefore we choose membranes to be the target to study the toxicity of oxygen free radicals. The
goal of our study is to answer the following questions:

1) To what extent are membranes vulnerable to attack by free radicals as compared to the cells from which they are derived?

2) What is the relative effectiveness of the individual radicals in producing damage to membranes?

3) By what mechanisms do they produce damage?

4) Does oxygen affect the damage to membranes caused by free radicals and by what mechanisms?

5) What are the interactions between different types of radicals when they are produced in a mixture?

6) How do such interactions modify the damage?

As with other diseases, advances in understanding of molecular mechanisms of free radical pathology are prerequisite to rational prevention, control and treatment of the disorders. The answers to these questions may enable
us to understand more about the characteristics of these radicals, their reactions with membranes and some of the mechanisms of their toxicity. In addressing these questions, we selected the plasma membranes of erythrocyte ghosts as the target for free-radical damage, and gamma-irradiated aqueous buffer as the source of potentially damaging free radicals, for the following reasons:

Resealed erythrocyte ghosts represent a suitable membrane system for this study, as they are devoid of cytoplasmic contents and resemble native plasma membranes in at least two important aspects: (a) they have the same composition of the major structural components of plasma membranes, namely lipids and proteins, (b) they possess permeability characteristics, similar to plasma membranes with respect to alkali ions and nonelectrolytes. These ghosts were prepared from erythrocytes by allowing the cytoplasmic contents to leak out during a brief osmotic rupture of the membranes. The leaking membranes can be resealed by incubation in isotonic buffer at 37 deg C for an hour. Resealing restores the characteristic permeability to alkali ions. However, during resealing some of the cytoplasmic contents, namely enzymes and low molecular weight molecules, are retained.
Organelles like nuclei and mitochondria are not found in ghosts; mature erythrocytes lack such organelles. As criteria for damage we selected (1) the activity of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as an oxidation sensitive SH-enzyme and (2) the permeability of the membrane to relatively low molecular weight substrates as an index of its functional integrity. Permeability can be conveniently measured by making use of the enzyme G3PDH, which is bound on the cytoplasmic side of the membranes. Since the resealed ghosts are impermeable to the substrates, NAD and G3P of this enzyme, detection of the activity of the enzyme towards substrates added externally indicates an increase of membrane's permeability, and therefore damage to the membrane.

In examining the actions of free radicals, it was necessary to make a choice among the various available methods of producing free radicals. Methods of producing \( \cdot \text{OH}, \text{O}_2^-, \) and \( \text{H}_2\text{O}_2 \) are discussed in detail in a recent symposium (Singh 1978). Of these methods, gamma-irradiation provides the simplest means of producing these radicals free from contaminating side-products. The breakdown of water by gamma radiation produces \( \cdot \text{OH}, \text{H}_2\text{O}_2, \cdot \text{H} \) and \( \text{e}^- \) as the major
products. In the presence of O₂, O₂⁻ is formed from H⁺ and e⁻. Each of these free radicals can be selectively produced in an aqueous solution by using radical scavengers to remove the other free radicals. Alternatively, all these radicals can be produced simultaneously. Therefore, effects of individual radicals and various mixtures of free radicals can be studied. Gamma-irradiation in combination with free radical scavengers was thus chosen in our study for its simplicity in application and versatility in producing free radicals. Furthermore, it is the most exhaustively studied system and its major free radical products are well established.

This thesis consists of 4 sections corresponding to papers in preparation for or submitted for publication.

In section 1 we show an ambivalent role of oxygen in either enhancing or protecting against free radical damage, and show membranes as one of the structures most sensitive to aggravation of damage by oxygen. This section was contributed mainly by Dr. Allan Davison and is intended for publication as a short note, reviewing our present knowledge of the contradictory effects of oxygen on free-radical
damage to biological systems. Detailed accounts of methodology are therefore deferred to section 2, and references to the original papers are provided. The author's contributions to this section are the data on damage to erythrocyte ghosts by irradiation and editing of the text.

In section 2 we summarize the methods used for preparing resealed ghosts from erythrocytes and for producing free radicals by gamma-irradiation under different concentrations of oxygen. Here we present evidence that oxygen unambiguously potentiates damage to plasma membranes by the free radicals studied, and that erythrocyte ghost cells are a suitable target for the study of free-radical induced damage.

In section 3 we describe the methods for isolating various free radical forms of oxygen, and compare the relative effectiveness of each in regard to two different criteria for radiation damage, namely the activity of membrane-bound G3PDH and permeability.
In section 4 we present results of an investigation of interactions among the various radicals when present in a mixture. We show that such interactions modify the damage sometimes synergistically, and sometimes by mutual quenching. The relative effectiveness of catalase, superoxide dismutase, formate and mixtures of these as radioprotective agents are also compared.
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FUNCTIONS OF OXYGEN IN FREE RADICAL DAMAGE TO BIOLOGICAL SYSTEMS: EVIDENCE FOR A DUAL ROLE AS BOTH SENSITIZER AND PROTECTOR

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Supported by grant #719113 from the Canadian Medical Research Council, the Simon Fraser University President's Research Fund, and the B.C. Department of Labour. We thank Dr. Jeffrey Bland of the University of Puget Sound for his advice on the technique of membrane preparation and constructive comments. The impeccable technical assistance of Ms S-Pong Khoo is acknowledged with gratitude.
Oxygen is known to mediate various kinds of free-radical damage in biological systems. However, in attempting to investigate IN VITRO its cellular role in free radical damage, several instances were found in which the presence of oxygen protects against free radical damage, and only one instance in which it increased damage. The free radical damage to catalase initiated by ascorbate was examined under atmospheres of 100% oxygen, air, or nitrogen. Either in the presence or absence of a catalytic copper(II) complex, damage was greatest anaerobically, least under oxygen, and intermediate in the presence of air. Consistent with this finding, superoxide was not implicated in the destructive process, using superoxide dismutase as a probe of its involvement. Similarly, the rate of haemolysis induced by pro-oxidant drugs was decreased by the presence of oxygen. Damage to cytochrome c by gamma-irradiation was studied at dosages ranging from 9 to 160 krads in the presence and absence of formate (5mM), lecithin (egg yolk) (5mg/100ml), and superoxide dismutase (1 nM) at various partial pressures of oxygen. A consistent phenomenon in these studies was an
increase in damage by up to 50% after the removal of oxygen under each of the reaction conditions studied, except in the presence of superoxide dismutase which in some cases blocked the protective effect of oxygen. In contrast to these three protective actions of oxygen, the only instance in which it was found to have a destructive action was in the damage to erythrocyte ghost cells induced by gamma-irradiation. We interpret these findings, together with supporting data from analogous chemical systems as evidence that oxygen, like other free radical reagents, may play an ambivalent role in both initiating or inhibiting, enhancing or retarding cellular free radical processes.
FUNCTIONS OF OXYGEN IN FREE RADICAL DAMAGE TO BIOLOGICAL SYSTEMS: EVIDENCE FOR A DUAL ROLE AS BOTH SENSITIZER AND PROTECTOR

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The well established role of oxygen in free radical damage to living organisms has been emphasized recently in reviews of oxygen toxicity (1) (2), of free radical pathology (3) (4) (5), of radiation damage (6) (7), of drug induced haemolytic anemias (8) (9), and of free radical damage to enzymes initiated by pro-oxidants (10) (11) (12).

Evolutionary implications of the hazards of atmospheric oxygen have been reviewed (13), also the presence of superoxide dismutase in all aerobic cells and its absence from obligate anaerobes (14) (15) provide evidence of the importance of protective mechanisms in living organisms.
Oxygen concentration in a target tissue is a major factor determining the severity of the damage produced by radiation (16). Postulated intermediates in radiation effects include the superoxide anion derived from the reaction of molecular oxygen with hydrogen atoms, hydrated electrons, perhydroxyl radicals etc. (17). Some radioprotective agents are thought to act as scavengers of these radical intermediates (7) while others act by decreasing local oxygen concentration, either through direct reaction with oxygen or by destroying the capacity of haemoglobin to transport oxygen (18). The enhancement of radiation damage by the presence of oxygen has been extensively documented in systems of various degrees of organization, for example: purified dry trypsin (19), the breakage of DNA single strands in mammalian cells (20), the survival of cultured mammalian cells (21) or Erlich ascites tumour cells (16), and the production of radiation myelopathy (22) (23).

In attempting to define the role of oxygen and the superoxide radical in free radical damage, we found ourselves forced to document and explain a contrasting protective action of oxygen when we found that the amount of damage was substantially greater in the absence of oxygen
than in its presence. This was observed in several only remotely related systems: gamma-irradiation of cytochrome c, ascorbate induced destruction of catalase, and pro-oxidant induced haemolysis. Discussion with other workers revealed that protective actions of oxygen against free radical damage probably occur more often than destructive effects IN VITRO. However, inhibition of free radical reactions by oxygen have been reported much less frequently, since these 'negative' results are often left unpublished. Raymond and Weintraub have reported that oxygen inhibits the polymerization of acrylamide initiated by free radicals (24), and Folkman and co-workers have confirmed these findings and utilized them in a clinical assay for oxygen in blood (25,26). The inhibition of haemoglobin autoxidation by saturation with oxygen is another example (with oxygen presumably playing a different role) which may be clinically exploited in the proposed use of hyperbaric oxygen in the treatment of methaemoglobinemia (27). Likewise, radiation damage is not always increased by the presence of oxygen (28). Despite the prevalence of the well established radiosensitizing effects of oxygen IN VIVO, the effects IN VITRO are not so clear cut. There have been at least two reports of protection IN VITRO by the presence
of oxygen in irradiated enzymes, namely trypsin (29) and chymotrypsin (30), and several reports that no effect of oxygen could be detected, e.g. in the cases of catalase (31), carboxypeptidase (32), or ribonuclease (33). These inconsistent effects of oxygen were explained (28) on the basis that radiation-activated oxygen destroys some enzymes but not others, and from the results of Hutchinson (34), on the basis that the presence of sulphydryl compounds in some enzyme preparations may be required for oxygen damage to occur.

Orr (10) has suggested that the destruction of catalase by ascorbate is mediated by the superoxide radical. We therefore examined the destruction of catalase in the presence of ascorbate at various oxygen concentrations and in the presence and absence of superoxide dismutase. In 100% oxygen, 19% oxygen and under anaerobic conditions the acceleration of destruction due to presence of ascorbate was 40, 240, and 720% respectively in the absence of copper(II) (Figure 1) and 110, 320, and 1100% in the presence of copper(II) acetate (Figure 2). Taken together with failure of superoxide dismutase to inhibit the damage, the results appear to exclude the postulated role of superoxide (10) as
a mediator of the damage while implicating oxygen as a scavenger of at least some of the radicals actually responsible for the damage.

In an attempt to assess the role of oxygen radicals in damage to biological membranes induced by pro-oxidant substances, a study was made of haemolysis induced by enediol and phenolic pro-oxidants. The rates of haemolysis and methaemoglobin formation induced by DOPA were compared in air and under an atmosphere of nitrogen. Neither haemolysis (Figure 3) nor methaemoglobin formation (Figure 4) was slowed by the removal of oxygen, rather acceleration of both was noted, haemolysis being about 250% faster under anaerobic conditions than in the presence of oxygen, and the formation of methaemoglobin about 300% faster. In contrast the addition of CO to the gas mixture to a concentration of 8% substantially inhibited the methaemoglobin formation, but without inhibiting haemolysis. Evidently, despite the protective role of oxygen, the interaction of some CO binding protein with oxygen is a prerequisite to the acceleration of haemoglobin autoxidation by the pro-oxidants. This protein might be haemoglobin or the erythrocyte's cytochrome b5-like protein. In either event a
dual role for oxygen as both promoter and inhibitor of the formation of methaemoglobin may be inferred from the results.

Damage to cytochrome c and to horseradish peroxidase by gamma-irradiation was studied at dosages ranging from 6 to 160 krads (Figure 5), in the presence and absence of formate, lecithin, and superoxide dismutase at various partial pressures of oxygen. Typical results at 60.5 rads dosage are given in Table 1. The presence of oxygen did not enhance destruction, whether in the presence or absence of either formate or lecithin. Instead, damage was consistently greater in the absence of oxygen except where superoxide dismutase was present. Adding formate to increase the superoxide yield provided a further increase in the protective effect of oxygen, while the anaerobic and delayed damage were both enhanced by the presence of formate. The addition of lecithin had no effect on the destruction occurring during irradiation, but increased the delayed damage 200 to 300%. The presence of superoxide dismutase increased the amount of aerobic damage, and in fact removed the ability of oxygen to protect.
In view of the vulnerability of biological membranes to lipid autoxidation, we examined the susceptibility of resealed erythrocyte ghost cells to doses of 30 to 100 krad of gamma-radiation, in the presence of oxygen, air, or nitrogen. As a measure of the integrity of the cell membrane we used both the activity of membrane-bound glyceraldehyde-3-phosphate dehydrogenase, and its accessibility to substrates added externally (Figures 6 and 7). In both cases damage was dramatically accelerated by the presence of oxygen, and almost complete protection was afforded by anaerobic conditions. Similarly, substantial protection was afforded by superoxide dismutase, catalase, or a mixture of the two.

In relation therefore to a variety of targets, we conclude that oxygen, and the superoxide radical, while sometimes destructive, more often had a paradoxical protective role, due to their ability to remove even more reactive free radicals. In many studies of a variety of enzymes it has been shown that hydroxyl radicals are the main damaging species during irradiation (35). Less reactive radicals therefore sometimes play a protective role. Powers, for example (36) has shown that aqueous electrons act as
protective agents by reacting with hydroxyl radicals: OH + e- → OH-. The simplest explanation of our data is that the superoxide radical protects against damage by hydroxyl radicals in an analogous manner:

\[ \text{O}_2^- + \cdot \text{OH} \rightarrow \text{O}_2 + \cdot \text{OH}^- \]

This reaction has been proposed to account for a suppression of the yield of superoxide under conditions in which formation of hydroxyl radicals is increased (37) during the irradiation of oxygenated water. The results presented here suggest the converse: i.e., under the conditions of our experiments, the presence of superoxide radicals suppressed the production and action of hydroxyl radicals. This view is supported by the finding that net formation of hydroxyl radicals in the presence of 6-hydroxydopamine may be decreased by the presence of superoxide radicals (38). Similarly McCord and Fridovich reported that superoxide dismutase increased the oxidation of ferrocytochrome c by hydroxyl radicals in photolyzed water, and they conclude that the superoxide radical acts as a scavenger of hydroxyl radicals (39).
In the irradiation of cultured cells, a dual role for oxygen as a radiosensitizing and radioprotective agent depending on the irradiation technique, has been described by Revesz (40) and explained in terms of a requirement for oxygen by the radiation repair mechanisms, an explanation not tenable for the protective action of oxygen in highly purified systems. Clearly the roles of oxygen radicals in radiation damage vary dramatically, depending on radiation dose and its administration, the nature of the damage being measured, the identity of the target substance, and the degree of organization of the system being studied.

For example, oxygen may act as a protective agent in those systems which are susceptible to damage by reducing agents by virtue of its ability to scavenge reducing free radicals such as electrons or hydrogen atoms. In other systems which are particularly susceptible to oxidative or reductive damage by hydroxyl radicals, oxygen may increase the yield of superoxide radicals, which in turn afford protection against hydroxyl radicals. On the other hand, in systems which are susceptible to lipid autoxidation, perhaps involving superoxide radicals, or other intermediates in Haber-Weiss like reaction, free radical species of oxygen
form an integral part of the free radical chain propagation mechanism, and oxygen is required for the maximum damaging effect.

Consequently, when oxygen is radiosensitizing in vivo, the commonly observed oxygen enhancement ratios of 2 to 3 may represent a balance between radioprotective and radiosensitizing effects, with some or all of the latter being accounted for by effects mediated by damage to membranes and membrane-bound proteins.

It is perhaps not surprising that relatively stable free radicals such as oxygen or the superoxide radical should act as inhibitors of processes which depend on more reactive radical intermediates and in some cases on chain propagation. Free radicals commonly play the dual roles of chain initiators and chain terminators and this property of oxygen is well known to polymer chemists. What is surprising is that this paradoxical property of oxygen remains more or less undocumented in the biological and biochemical literatures. In the light of the experimental data reported here, a role for oxygen in cells as a potential free radical protective agent or even as an
antioxidant under some circumstances should be considered, with the superoxide radical as a mediator of this role in some cases.
BIBLIOGRAPHY


FIGURE 1. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF CATALASE BY ASCORBATE. Conditions were: 0.1 M phosphate buffer pH 7.0, temperature 25 deg C, catalase concentration 10uM, ascorbate concentration 10 mM. In each case, atmospheres of nitrogen (Linde high-purity grade, less than 5 parts per million oxygen), or oxygen (Linde high-purity grade) were induced in rubber capped vials using a Virtis gas manifold, by five cycles of repeated evacuation to boiling, and reintroduction of the desired gas to a slight positive pressure.

Symbols

Control
Nitrogen
Air
Oxygen
FIGURE 1. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF CATALASE BY ASCORBATE.
FIGURE 2. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF CATALASE BY ASCORBATE IN THE PRESENCE OF COPPER. Conditions were: 0.1 M phosphate buffer pH 7.0, temperature 25 deg C, catalase concentration 10 uM, ascorbate concentration 10 mM, copper(II) acetate concentration 5 mM.

Symbols

Control ▲
Nitrogen ○
Air ▼
Oxygen △
FIGURE 2. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF CATALASE BY ASCORBATE IN THE PRESENCE OF COPPER.
FIGURE 3. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE RATE OF HAEMOLYSIS INDUCED IN WASHED ERYTHROCYTES BY THE PRESENCE OF 5 mM DOPA. Conditions were: 0.01 M phosphate buffer pH 7.0 rendered isotonic by the addition of NaCl to 300 m0sm, temperature 25 deg C, concentration of cells 5 % v/v.

Symbols

\begin{itemize}
  \item Nitrogen \( \odot \)
  \item Air \( \square \)
  \item Oxygen \( \triangle \)
\end{itemize}
OF HAEMOLYSIS INDUCED IN WASHED ERYTHROCYTES BY THE PRESENCE OF 5 mM DOPA.
FIGURE 4. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE FORMATION OF METHAEMGLOBIN, INDUCED IN WASHED INTACT ERYTHROCYTES BY THE PRESENCE 5 mM DOPA. Conditions were: 0.01 M phosphate buffer pH 7.0 rendered isotonic by the addition of NaCl to 300 mosM, temperature 25 deg C, concentration of cells 5% v/v.

Symbols

Nitrogen
Air
Oxygen

O
II
A
FIGURE 4. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE FORMATION OF METHAEMOGLOBIN, INDUCED IN WASHED INTACT ERYTHROCYTES BY THE PRESENCE 5 mM DOPA.
FIGURE 5. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF CYTOCHROME C BY GAMMA-IRRADIATION.

Conditions were: 0.1 M phosphate buffer pH 7.0, temperature 23.5 deg C, cytochrome C concentration 30 µM.

Symbols

Nitrogen  ○
Air  ●
Oxygen  △
FIGURE 5. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF CYTOCHROME C BY GAMMA-IRRADIATION.
FIGURE 6: EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF THE PLASMA MEMBRANE OF RESEALED ERYTHROCYTE GHOST CELLS BY GAMMA-IRRADIATION AT A RATE OF 0.5 MRAD/MIN. Conditions were: 0.01 M phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mOsM, temperature 25 deg C, concentration of ghosts 10% v/v.

Symbols

- Control
- Nitrogen
- Air
- Oxygen
FIGURE 6. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF THE PLASMA MEMBRANE OF RESEALED ERYTHROCYTE GHOST CELLS BY GAMMA-IRRADIATION AT A RATE OF 0.5 KRAD/MIN.
FIGURE 7. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF MEMBRANE-BOUND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF RESEALED ERAYTHROCYTE GHOST CELLS BY GAMMA-IRRADIATION AT A RATE OF 0.5 K RAD PER MIN. Conditions were: 0.01 M phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mosm, temperature 25 deg C, concentration of ghosts 10 % v/v.

Symbols

Control  ○  □
Nitrogen  ⊕
Air  △  ⊗
Oxygen  □
FIGURE 7. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE DESTRUCTION OF MEMBRANE-BOUND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF RESEALED ERYTHROCYTE GHOST CELLS BY GAMMA-IRRADIATION AT A RATE OF 0.5 KRAD PER MIN.
TABLE 1. EFFECTS OF OXYGEN CONCENTRATION ON THE RATE OF DESTRUCTION OF CYTOCHROME C DURING GAMMA-IRRADIATION, IN THE PRESENCE OF FORMATE, SUPEROXIDE DISMUTASE, OR LECITHIN.

Values given are uM cytochrome c destroyed per minute during 60.5 krad of radiation given over a period of one hour, based on the oxidised/reduced difference spectrum at 550 nm. Conditions were: 0.1 M phosphate buffer pH 7.0, temperature 23.5 deg C, cytochrome c concentration 30 uM. Formate when present was at 5 mM, superoxide dismutase at 0.82 nM, and lecithin at 0.005% final concentration in the reaction mixture.
<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>RATE OF DESTRUCTION OF CYTOCHROME C DURING IRRADIATION (μM·min⁻¹)</th>
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<tr>
<td></td>
<td>Oxygen</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.0965 ± 0.012</td>
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<tr>
<td>Cytochrome c + superoxide dismutase</td>
<td>0.147 ± 0.018</td>
</tr>
<tr>
<td>Cytochrome c in formate</td>
<td>0.0379 ± 0.005</td>
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<tr>
<td>Cytochrome c in formate + superoxide dismutase</td>
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<tr>
<td>Cytochrome c + lecithin</td>
<td>0.105 ± 0.013</td>
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</table>
ACTIONS OF GAMMA RADIATION ON RESEALED ERYTHROCYTE GHOSTS.
A COMPARISON WITH INTACT ERYTHROCYTES AND A STUDY OF THE
EFFECTS OF OXYGEN

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Resealed erythrocyte ghosts proved a convenient biological target for investigation of the effects of various free radical forms of oxygen on plasma membranes. The activity of membrane-bound G3PDH provided a means for assessing permeability of ghosts as well as for providing additional information on damage to membrane-bound proteins. With respect to permeability and inactivation of membrane-bound G3PDH, ghosts were more susceptible than erythrocytes to free radicals produced in the gamma-irradiation of aqueous solutions. The permeability of irradiated ghosts was immeasurably greater than that of irradiated erythrocytes, while inactivation of G3PDH was 21-fold greater. The sensitivity of ghosts to radiation damage was affected strongly by the presence of oxygen during irradiation. In the presence of air, the rates of increase in permeability and inactivation of G3PDH were 2.8- and 1.5-fold those in the presence of N2. The use of buffer saturated with oxygen accelerated the aerobic rates of increase in permeability and inactivation of G3PDH by 60- and 2.7-fold respectively. These results indicate that inactivation of G3PDH is relatively insensitive to oxygen, while permeability is very
sensitive to oxygen, particularly at high concentrations of oxygen. However, the rate of inactivation of G3PDH was an order of magnitude greater than that of the increase in permeability, indicating that the former is much more sensitive to irradiation. The sensitivity of irradiated ghosts to oxygen indicates that plasma membranes are at least one of the loci at which oxygen enhances radiation damage to living cells. The major mechanism of this "oxygen effect" is the ability of oxygen to increase the branching of free radical chain reactions propagating damage after their initiation within the membrane.
1. Introduction.

Results from studies of irradiation of living cells or tissues (Koch, Kruuv and Frey 1973, Ewing 1978) are difficult to interpret due to the complexity of the cellular system. In addition, the protective effects of catalase (Fee and Bergamini 1975, Rapoport 1974), superoxide dismutase (Zimmerman, Flohe, Weser and Hartmann 1973, Petkau and Chelack 1976, Petkau, Kelly, Chelack and Barefoot 1976, Fee and Teitelbaum 1972), glutathione (Quitiliani 1977) and glutathione peroxidase (Nicholls 1972) in the cytoplasm further complicate interpretation of the results. Since the cytotoxic effects of irradiation are largely due to substrates of these enzymes (Michaels and Hunt 1978), these compounds protect the cell by removing the free radicals which are produced in the radiolysis of water. The free radicals produced are e-, .OH, H2O2, and H. In the presence of oxygen, e- and .H will be converted to O2- (Draganic, Kuzadovic and Draganic 1969). In a previous paper in this series we described several investigations of the effects of oxygen on isolated biological systems in which, far from aggravating radiation damage, the presence of oxygen was protective (Kong 1979).
Clearly, a better understanding of the mechanisms of participation of oxygen in cellular radiation damage is needed and should be obtainable from studies of irradiation of individual cellular components. The functional integrity of the plasma membranes is vital to the cell's existence and the unsaturated fatty acids found in membranes are known to be susceptible to damage by free radicals (Raleigh, Kremer and Gaboury 1973, Zimmermann, Flohe, Weser and Hartmann 1973, Wills and Wilkinson 1967, Wills and Rotblat 1965). Therefore, it is reasonable to expect membranes to be one of the important sites in the cell at which damage by free radicals may explain the toxic effects of irradiation. Biological membranes are critical targets in irradiated cells (Alper 1968) and major loci for the development of oxygen effect (Alper 1971). Alper found that damage to DNA irradiated outside cells was not enhanced by the presence of oxygen. In contrast, o.e.r. (oxygen enhancement ratio) of damage to DNA irradiated intracellularly was 1.3. He proposed that the membrane is necessary for the enhancement of damage to DNA by oxygen. Furthermore, the o.e.r.'s for the killing of mammalian cells are also commonly higher than can be accounted for by DNA damage whereas the o.e.r. for damage to membranes was sufficiently high. Free-radical
damage to membranes has also been demonstrated by Bland (1975, 1978), by Popma (1974) and Davison and Popma (1978). These studies showed that free radicals caused oxidation of lipid which was related to the lysis of the membranes.

The susceptibility of plasma membranes to damage by free radical forms of oxygen has been of particular interest, because of the existence of various haemolytic anaemias induced by pro-oxidant drugs, vitamin E deficiency, or deficiency of one or other of the antioxidant enzymes (Jacob and Jandl 1962a, 1962b, Allen and Jandl 1961, Fee, Bergamini and Briggs 1975). In each case the haemolysis is thought to be due to attack on the plasma membrane by one or more of the free radical forms of oxygen, but particularly by superoxide, hydrogen peroxide and hydroxyl radicals resulting from an interaction between the first two.

Ghosts prepared from erythrocytes offer a near ideal membrane system which contains little or no cytoplasmic contents and the biochemical and structural characteristics of erythrocyte ghosts are well established (Hoffman 1958, Schrier 1963, 1966, Hanahan 1973, Tavera and Langdon 1973, Zwaal, Roelofsen and Colley 1973, Tillmann, Cordua and
Schroter 1975, Podan, Podan and Sha'afi 1976, Yung, Carlson and Balzer 1972). Of the different types of ghosts prepared from erythrocytes, resealed ghosts bear close resemblance to the plasma membranes of erythrocytes in at least two important aspects: (a) they have the same composition of the major structural components of plasma membranes, namely lipids and proteins, (b) they possess permeability characteristics similar to plasma membranes with respect to alkali ions and nonelectrolytes (Staros, Haley and Richards 1974, Schwoch and Passow 1973). Furthermore, the membrane-bound G3PDH provides a convenient measure of changes in permeability of the membrane, as well as a measure of damage to this membrane-bound protein.

For the above reasons we elected to examine the susceptibility of these characteristics of resealed ghosts to radiation damage, to compare the ghosts with intact erythrocytes, and to examine the role of oxygen in free radical damage to the plasma membrane. In this study, we will define conditions for the preparation and irradiation of ghosts, and show that ghosts are particularly sensitive to radiation and to the concentration of oxygen present during irradiation. Our results demonstrated that resealed
ghosts are a suitable membrane system to investigate free-radical damage, especially the cytotoxic effect of oxygen. In subsequent studies we have further investigated the role of oxygen in promoting radiation damage in this system with respect to both the relative effectiveness of individual free radicals and the role of interactions between them in the mechanism of the "oxygen effect".
2. Experimental

2.1 Materials

Glyceraldehyde-3-phosphate (G3P), dithiothreitol (DTT), Triton X-100 and beta-NAD were supplied by Sigma Chemicals Ltd. Sodium arsenate, sodium pyrophosphate, of A.C.S. standard were supplied by Fisher Chemicals Ltd.

2.2 Methods

2.2.1. Ghost preparation.

Recently outdated human blood was obtained from the Vancouver Red Cross blood bank, stored in citrate-phosphate-dextrose buffer. Resealed ghosts were prepared according to the method of Steck and Kant (1974) with the following modifications: 0.01 millimolar CaCl₂ and 4 millimolar MgCl₂ were included in the hypotonic buffer which had a final tonicity of 40 milliosmolar (pH 7.4). Erythrocytes were haemolysed in hypotonic buffer at 4 deg C, during which most of the cytoplasmic contents were released from the cells. The membranes were then resealed by
incubation in isotonic buffer at 37 deg C for 60 minutes. Such resealing restored the characteristic permeability to alkali ions. It was found that ghosts prepared in the presence of these low concentrations of Ca2+ and Mg2+ retained a smooth and biconcave surface and were more resistant to fragmentation (Bramley and Colman 1972, Duchon and Collier 1971). Also, when ghosts were prepared at 40 milliosmolar concentrations, most of them would remain intact. At lower osmolarity, fragmentation takes place (Bramley, Coleman and Finean 1971). Therefore, the modification ensured a higher yield of resealed ghosts. Ghosts prepared under these conditions, however, retained more hemoglobin (Dodge, Mitchell and Hanahan 1963). We have estimated the concentration of residual haemoglobin to be 10% ± 5% mean corpuscular haemoglobin (Popma 1974). The activities of residual catalase and dismutase were estimated to be 10*4 units and 37 units per ml of packed ghosts which are 12% and 8.7% of the activities in red blood cells.
2.2.2. Determination of permeability of the plasma membrane

Membrane permeability was determined by measuring the accessibility of the membrane-bound glyceraldehyde-3-phosphate dehydrogenase (G3PDH), found on the cytoplasmic side of ghosts to its externally added substrates. The resealed ghosts were normally impermeable to the substrate G3P and NAD. Therefore when these were added externally, an increase in total G3PDH activity of the ghost suspension indicates the extent of any increase in permeability. Using Triton X-100 to solubilise the membrane, the total G3PDH activity of the suspension can be measured from the rate of increase in absorbance at 340 nm. The percentage of ghost suspension which is permeable to G3P is given by the ratio of G3PDH activity in the absence of Triton X-100 to that in the presence of Triton X-100, thus:

\[
\text{G3PDH activity (} \text{+ Triton)} - \text{G3PDH activity (} \text{- Triton)} = 100\% - \% \text{ permeable ghosts}
\]

% permeable ghosts = \( \frac{\text{G3PDH activity (} \text{+ Triton)} - \text{G3PDH activity (} \text{- Triton)}}{\text{G3PDH activity (} \text{+ Triton)} } \times 100\%
\]

% intact ghosts = 100% - % permeable ghosts
2.2.3. Determination of the activity of membrane-bound G3PDH

G3PDH was assayed according to the method of Steck and Kant (1974) except that DTT was used instead of cysteine HCl. The assay made use of the reduction of NAD to NADH by G3P catalysed by G3PDH. The reaction was followed by the increase in absorbance (A) at 340 nm which is due to NADH. The assays were performed on a Beckman DB spectrophotometer at 25 deg C, and the course of reaction was recorded on a Beckman 10 inch strip chart recorder. The activity of the enzyme was calculated from the rate of increase of absorbance between the first and second minute of the reaction. The percentage of active enzyme of the irradiated samples was obtained from the ratio of the remaining enzymic activity of the solubilised membranes at any given dose to that at zero dose.

\[
\text{G3PDH activity (+Triton) at any dose} = \frac{\text{G3PDH activity (+Triton) at zero dose}}{\text{G3PDH activity (+Triton) at zero dose}} \times 100\%
\]
2.2.4. Irradiation conditions

The radiation source was a GAMMACELL 200 (Atomic Energy of Canada Ltd.). The dose rate was 0.5 krad/min. \((3.13 \times 10^{19} \text{ ev. 1-min}^{-1})\). Irradiation was performed at 25 deg C. Ghost suspensions containing 10% v/v ghosts were stored in 40 ml glass tubes containing 40 ml isotonic buffer and radical scavengers.

2.2.5. Induction of atmospheres other than air

For atmospheres other than air, the following procedure was used to saturate samples with N₂ or O₂ for irradiation: 40 ml samples of isotonic buffer containing dissolved free radical scavengers were sealed in glass tubes with rubber caps. Membranes were added only after flushing was finished to avoid frothing. Although catalase or superoxide dismutase was present in the buffer, frothing was negligible, due to the low concentrations of these proteins. A short narrow bored needle was inserted through the rubber cap until it was above the meniscus. The needle was connected to a suction pump by tygon pressure tubing. Deaeration of the buffer was carried out until no more
bubbling was observed. The tubing was then removed and a second long needle was inserted until its tip was just above the bottom of the glass tube. Nitrogen or oxygen was supplied slowly to maintain a steady slow rate of bubbling for 20-30 min. At the end of such flushing, the short needle was withdrawn before the gas was turned off to leave a slight positive pressure inside the tube. After the second needle was withdrawn, membranes were added by a syringe and the tube was shaken to mix the suspension. The sample was then ready for irradiation. Leakage could be detected if bubbling was observed when the cap was wet with a small quantity of water. The nitrogen used was 'ultra-high purity' from Linde, and contained less than 2 ppm oxygen. However, since the membranes were air-equilibrated before introduction into the anaerobic media, "anaerobic" in these experiments implies a residual oxygen concentration of about 25 micromolar. Oxygen concentration diminishes progressively during irradiation, and solutions containing oxygen initially at 25 (anaerobic), 230 (aerated) and 1150 (oxygenated) micromolar would reach values of 0, 236 and 1136 micromolar after about 8 minutes.
2.2.6. Analysis of data

Each of the slopes of the graphs in Figures 1 to 4 were obtained by regression analysis of 18 experimental observations representing on the average 3 separate experiments. The regression analysis was carried out by linear regression using the University of Alberta APL statistical package "STP2". The results were further confirmed using the APL program "AMCOVA1" based on the methods described in Sokal and Rohlf (1969) for analysis of covariance. The standard errors of the slopes of graphs of permeability as a function of time are on the average less than +/-30% while those of enzymic inactivation are less than +/-20%. The asymmetry of the gamma-source, the inevitable variation in the cytoplasmic contents and tocopherol content of the membrane preparations are probably the major sources of errors that account for the standard deviations of our data. However, these effects would be small in comparison to the effects of free radicals since irradiation in the absence of externally added scavengers produced significant damage to the membranes.
3. Results.

Erythrocytes irradiated in air-saturated buffer showed no change in permeability up to a dose of 150 krads. They were as stable as unirradiated erythrocytes or ghosts (Figure 1) which were incubated in air-saturated buffer at 25 deg C. In contrast, ghosts irradiated in air-saturated buffer showed a marked increase in permeability. This greater sensitivity of ghosts to irradiation than erythrocytes is reflected in the fact that the rate of increase in their permeability was many times that of unirradiated ghosts (Table 1). Erythrocytes are resistant to irradiation also with respect to inactivation of G3PDH. There was only a slight loss of activity of G3PDH in comparison with unirradiated ghosts (Figure 2). The rate of inactivation of G3PDH was 22-fold greater in ghosts ($8.43 \times 10^{-3}$ per hour) than in erythrocytes ($3.9 \times 10^{-3}$ per hour) irradiated under the same aerobic conditions (Table 1).

Ghosts exposed to different concentrations of oxygen during irradiation showed marked differences in both permeability and enzymic inactivation (Figures 3 and 4). The least damage was observed under anaerobic conditions while the
most damage was observed in the presence of 1 atmosphere of oxygen. Rates of inactivation of G3PDH were an order of magnitude greater than rates of increase in permeability in all cases except in oxygenated buffer (table 1), indicating a greater sensitivity of this enzyme to radiation than membrane permeability. The sensitivity of permeability and G3PDH to concentrations of oxygen can be compared using data in Table 1. The rates of increase in permeability of ghosts irradiated in the presence of air, and in the presence of 1 atmosphere of oxygen are respectively 2.8- and 170-fold that in the presence of nitrogen. The corresponding ratios in the case of inactivation of G3PDH are 1.5 and 4 respectively. The greater difference between the oxygen enhancement ratios in the case of permeability indicates that an increment of oxygen is far more effective in increasing rate of change of permeability than in accelerating inactivation of membrane-bound G3PDH. This greater sensitivity of membrane permeability to oxygen is better illustrated by the observation that the aerobic rate of increase in permeability is increased 60-fold by the increment in concentration of oxygen while that of inactivation of G3PDH is only increased by 2.7-fold. Therefore, enzymic inactivation is relatively insensitive to
oxygen compared to permeability but more sensitive to irradiation. An autocatalytic component in damage to permeability is apparent in the presence of oxygen, as indicated by the incipient curvature of the plot in Figure 3. The absence of such a phenomenon in the presence of air suggests that a certain threshold concentration of oxygen is required to precipitate an autocatalytic breakdown of the structural integrity of membranes.

The effect of oxygen on ghosts in the absence of radiation was further investigated in a control study in which ghosts were incubated in oxygen-saturated buffer at 25 deg C. Permeability and loss of enzymic activity of these ghosts were found to be only slightly greater than the control (i.e., ghosts incubated in buffer at 25 deg C in the presence of air) (Figures 3 and 4). Thus, the simultaneous presence of both oxygen and gamma-irradiation are required for the synergism displayed.
4. Discussion.

4.1. Comparison of erythrocytes and ghosts

In addition to the substantial amount of enzymes (catalase, superoxide dismutase, glutathione peroxidase and methaemoglobin reductase) that protect the red blood cells from oxidative damage, the abundance of organic molecules in the cells also aids in scavenging free radicals in a nonspecific manner, thereby protecting the membranes. The ghosts have lost most of their cytoplasmic contents during hypotonic hemolysis and furthermore the alteration of the native structure of the membranes due to the osmotic shock weakens the hydrophobic interactions in the membrane (Woodward and Zwaal 1972). Both of these could contribute to the greater susceptibility of ghosts towards radiation damage. IN VIVO, red blood cells must last over 100 days, so even slow rates of damage may be quite unacceptable. The high concentration of catalase and superoxide dismutase in the red blood cells are evidently necessary for protection against oxidative stresses, since the low concentrations of these enzymes remaining in our ghosts did not provide complete protection against radiation. However, in the
absence of pro-oxidant stresses, the relative stability of ghosts indicates that these residual enzymes are adequate to cope with the ongoing fluxes of free radicals even in the presence of pure oxygen (Figures 3 and 4).

4.2. Effects of concentrations of oxygen

Ghosts irradiated under different concentrations of oxygen showed marked differences in damage to permeability and G3PDH. The magnitude of damage to membranes is similar to that observed in studies of mammalian cells (Koch, Kruuv and Frey 1973). The increase in damage with increasing concentration of oxygen indicated the familiar oxygen enhancement of radiation damage (Ewing 1978, van Hammen, Meuling and Bleichrodt 1978, Yuhas and Li 1978, Howard-Flanders 1960).

The greater radiation resistance of permeability in comparison with G3PDH at moderate concentration of oxygen (i.e., in air-saturated buffer) can be explained as follows. More sites of damage by free radicals are probably required to produce an observable change in permeability. The protective features provided by the architecture of membrane
to limit spreading of chain reactions within the membrane is probably quite important in preserving the membrane under moderate oxidative stresses (Demopoulos 1973b). Proteins are among the important components in this protective mechanism. The proteins interrupt the midzone of the lipid bilayers and the aromatic amino acid residues, which are abundant in this region, can delocalise the unpaired electrons resulting from abstraction or addition in chain propagating sequences of reactions. Thus the chain reactions that propagate damage deep into the membrane may be slowed or terminated. Hydrogen bonding within the membrane also indirectly protects some molecular sites against hydrogen abstraction by free radicals. Ghost membranes, unlike mitochondrial membranes, are devoid of iron-sulphur proteins which are potent catalysts for lipid autoxidation (Hanstein and Hatefi, 1970). Results from studies using chaotropic agents suggest that the lipophilic region of the membranes is protected from attack by oxygen in solution (Hatefi and Hanstein 1970). In addition, vitamin E in the membranes acts as an antioxidant (Tappel 1973). The above explanations are reasonable in view of the facts that \( \cdot \text{OH}, \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) are known to cause lipid peroxidation in membranes (Stock and Dormandy 1971, Wills
and Wilkinson 1967, Wills and Rotblat 1965) and that the parallel increase in lipid peroxidation and permeability of ghosts reported by our laboratory (Popma 1974, Davison and Popma 1978) suggested a direct relationship between lipid peroxidation and the increase in permeability of ghosts.

That autocatalysis is observed only in the damage to permeability and not to G3PDH may reflect the continuous nature of membrane and the intimate relation between the lipids being necessary for the development of the oxygen effect. In contrast, the isolated nature of molecules of G3PDH in the membrane has partly prevented autocatalysis. It was further supported by the observations that the autocatalytic increase in permeability was observed also in oxygenated buffers containing scavengers (Kong 1973), but again it was not observed in enzymic inactivation. The ambivalent role of the molecular architecture of the membrane in providing an antioxidant effect at low concentration of oxygen and a facilitatory effect to oxidative damage at high concentration of oxygen can thus explain its ability in propagating the following chain reactions (Tappel 1973):
Chain reaction:

\[ \text{L.} + \text{O}_2 \rightarrow \text{LOO.} \] \hspace{1cm} (1)

\[ \text{LOO.} + \text{LH} \rightarrow \text{LOOH} + \text{L.} \] \hspace{1cm} (2)

Chain branching:

\[ \text{LOOH} \rightarrow \text{L.} + \cdot \text{OH} \] \hspace{1cm} (3)

\[ \text{L.} + \text{LH} \rightarrow \text{LOH} + \text{L.} \] \hspace{1cm} (4)

\[ \cdot \text{OH} + \text{LH} \rightarrow \text{H}_2\text{O} + \text{L.} \] \hspace{1cm} (5)

Since the rate of reaction (1) between the radical formed in the lipids and dissolved O2 depends on the concentration of O2 in the membrane, a high concentration of O2 markedly increases the overall rate. In oxygen-saturated solutions, the concentration of O2 in the membrane would be higher than in the aqueous phase, since the solubility of O2 is 7-8 times higher in nonpolar than in polar media. Furthermore, branching in the chain reaction due to spontaneous decomposition of the unstable LOOH produced would multiply
the number of free radicals dramatically, thus increasing the number of chains (Demopoulos 1973a). Of the two radicals formed in (3), LO, could only propagate laterally within each lipid layer, (Tappel 1973), but .OH being mobile, could initiate chains in any direction, thus effectively spreading peroxidation throughout the membrane. The extreme vulnerability of biological systems to .OH may be explained in terms of the failure of the protective systems built into the membrane's architecture, since .OH being mobile can propagate chain reactions longitudinally. From the foregoing, it can be seen that O2 accelerates lipid oxidation by allowing and accelerating the formation of lipid hydroperoxides, thus increasing the number and length of the ensuing chain reactions. At low concentration of O2, termination of the chains may compete more effectively with reaction (1). The concentration of LOOH would be low due both to lack of availability of O2, as well as to the increased chain termination. Thus in anaerobic solutions, the propagation of peroxidation due to LO would still operate but chain branching would not occur. Hence the difference in damage between oxygen-saturated solutions and solutions of lower oxygen concentration is due to differences both in length and in number of chain reactions.
within the membrane. Our observations support such a mechanism. First, the low initial rate followed by an increasing rate is characteristic of branching free radical chain reactions. Second, oxygen is known to act both as a chain initiating and as a terminating species in chain reactions (Purdie 1971, Huyser 1970, Davison and Kaminsky 1974). The very low initial rates of increase of permeability by the mixture of radicals in the presence of oxygen could be due to termination of chain reactions by oxygen. Furthermore lipid peroxidation was found to be independent of the presence of oxygen during peroxide formation initiated by gamma radiation, but the subsequent formation of peroxide increased markedly if oxygen was present in the post-irradiation period (Wills and Wilkinson 1967).

The mechanism of oxygen enhancement of damage to membrane-bound G3PDH may be quite different, as indicated by the more linear damage shown in Figure 4. Damage due to reactive species such as lipid peroxides arising in the membrane and thus co-oxidation cannot be excluded in view of the severe damage to membrane in the presence of oxygen. From studies on irradiated sulphydryl compounds, it was
found that the major products formed in the presence of oxygen were sulfinic and sulfonic acids (Purdie 1971) which were responsible for the inactivation of sulphydryl enzymes (Buchanan 1978, Lin 1978). Therefore oxygen might increase damage to membrane-bound G3PDH by increasing the formation of these acids.

These results demonstrate not only that ghosts constitute a convenient and sensitive system for investigation of effects of radiation on plasma membranes, but more importantly, that membranes are one of the few identified loci for the origin of the "oxygen effect" observed in irradiated cells.
TABLE 1. EFFECTS OF CONCENTRATIONS OF OXYGEN ON RATES OF DAMAGE TO MEMBRANE-BOUND G3PDH AND MEMBRANE OF ERYTHROCYTES AND GHOSTS. The rates of increase in permeability are obtained from the slopes in Figure 1. The rates were expressed in log per cent of intact ghosts remaining after each hour of irradiation. The rates of inactivation of G3PDH were calculated from the slopes in Figure 2. The rates were expressed in log per cent activity remaining after each hour of irradiation. Conditions were: 0.01 M phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mOsM, temperature 25 deg C, concentration of ghosts or erythrocytes 10%/v, dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffer solutions air-saturated by atmospheric air unless oxygen or nitrogen is specified. The negative rates indicated no significant damage. The slopes and their standard errors were obtained by regression analysis (see Methods). The standard errors of the rates of increase in permeability and enzymic inactivation are on the average less than +/-30% and +/-20% respectively.
<table>
<thead>
<tr>
<th></th>
<th>Rate of increase in permeability x 10^-3</th>
<th>Rate of inactivation of G3PDH x 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unirradiated ghosts in buffers:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air-saturated</td>
<td>0.97 +/-1.6</td>
<td>-4.11 +/-6.01</td>
</tr>
<tr>
<td>Oxygenated</td>
<td>1.66 +/-2.26</td>
<td>0.97 +/-1.48</td>
</tr>
<tr>
<td><strong>Irradiated ghosts in buffer:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air-saturated</td>
<td>16.45 +/-3.15</td>
<td>84.28 +/-7.99</td>
</tr>
<tr>
<td>Oxygenated</td>
<td>2.75 +/-3.29 (initial)</td>
<td>119.5 +/-16.5 (initial)</td>
</tr>
<tr>
<td></td>
<td>986 +/-223 (final)</td>
<td>229.8 +/-11.6 (final)</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>5.73 +/-0.67</td>
<td>55.5 +/-7.62</td>
</tr>
<tr>
<td><strong>Erythrocytes irradiated in air-saturated buffer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.10 +/-0.36</td>
<td>3.94 +/-2.92</td>
</tr>
</tbody>
</table>
FIGURE 1. COMPARISON OF THE EXTENT OF RADIATION DAMAGE TO PERMEABILITY OF ERYTHROCYTES AND GHOSTS IN THE PRESENCE OF AIR. Log per cent ghosts of erythrocytes remaining intact after each hour of irradiation was plotted against time in hour. Conditions were: 0.01 M. phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mOsm., temperature 25 deg C, concentration of ghosts or erythrocytes 10% v/v, dose rate of gamma-irradiation 0.5 krad/min. dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffer solutions air-saturated by atmospheric air unless oxygen or nitrogen is specified. The slopes and their standard errors were obtained by regression analysis (see Methods).

Symbols

- **Unirradiated ghosts**
- **Unirradiated ghosts + O2**
- **Irradiated ghosts**
- **Irradiated erythrocytes**
FIGURE 1. COMPARISON OF THE EXTENT OF RADIATION DAMAGE TO PERMEABILITY OF ERYTHROCYTES AND GHOSTS IN THE PRESENCE OF AIR.
FIGURE 2. COMPARISON OF THE EXTENT OF RADIATION DAMAGE TO ACTIVITY OF G3PDH OF ERTHROCYTES AND GHOSTS IN THE PRESENCE OF AIR. Log per cent G3PDH remaining active after each hour of irradiation was plotted against time in hour. Conditions were as described in Figure 1.

Symbols

- Unirradiated ghosts
- Unirradiated ghosts + O2
- Irradiated ghosts
- Irradiated erythrocytes
FIGURE 2. COMPARISON OF THE EXTENT OF RADIATION DAMAGE TO ACTIVITY OF G3PDH OF ERYTHROCYTES AND GHOSTS IN THE PRESENCE OF AIR.
FIGURE 3. EXTENT OF RADIATION DAMAGE TO PERMEABILITY OF GHOSTS AT DIFFERENT CONCENTRATIONS OF OXYGEN. Log per cent ghosts remaining intact after each hour of irradiation was plotted against time in hour. Conditions were as described in Figure 1. Unirradiated ghosts incubated at 25 deg C in air-saturated buffer were used as control. Unirradiated ghosts were also incubated in oxygen-saturated buffer to monitor effect of oxygen in the absence of irradiation.

Symbols

- Nitrogen
- Air
- Oxygen
FIGURE 3. EXTENT OF RADIATION DAMAGE TO PERMEABILITY OF GHOSTS AT DIFFERENT CONCENTRATIONS OF OXYGEN.
FIGURE 4. EXTENT OF RADIATION DAMAGE TO ACTIVITY OF G3PDH OF GHOSTS AT DIFFERENT CONCENTRATIONS OF OXYGEN. log per cent G3PDH remaining active after each hour of irradiation was plotted against time in hour. Conditions were as described in Figure 3.

Symbols

Nitrogen   
Air    
Oxygen   

76
FIGURE 4. Extent of radiation damage to activity of G3PDH of ghosts at different concentrations of oxygen.
BIBLIOGRAPHY


SECTION 3

THE ROLES OF INDIVIDUAL FREE RADICALS IN THE EFFECTS OF
GAMMA-IRRADIATION ON THE PERMEABILITY OF MEMBRANES AND
ACTIVITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF
RESEALED ERYTHROCYTE GHOSTS

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constructive comments.
ABSTRACT

The relative effectiveness of oxidizing (\(\cdot OH, H_2O_2\)) and ambivalent (\(O_2-\)) and the reducing free radicals: (\(e-\) and \(CO_2-\)), in causing damage to membranes and membrane-bound G3PDH of resealed erythrocyte ghosts has been determined.

Rates of damage to membrane-bound G3PDH (\(R(\text{enz})\)) were measured and the rates of damage to membranes (\(R(\text{mb})\)) were assessed by measuring changes in permeability of the resealed ghosts to the relatively low molecular weight substrates of this enzyme. Each of the radicals was selectively isolated from the mixture produced during gamma-irradiation, using appropriate mixtures of scavengers such as catalase, superoxide dismutase and formate. \(\cdot OH\), \(O_2-\) and \(H_2O_2\) were approximately equally effective in inactivating membrane-bound G3PDH while, \(e-\) and \(CO_2-\) were the least effective. \(R(\text{enz})\) for \(O_2-\) and \(H_2O_2\) were 10-fold and for \(\cdot OH\) 15-fold, that of \(e-\). \(R(\text{mb})\) values were quite similar for \(e-\) and \(H_2O_2\); about twice that of \(O_2-\) while that for \(\cdot OH\) is 3-fold that of \(O_2-\). Hence, with respect to \(R(\text{mb})\): \(\cdot OH > e- = H_2O_2 > O_2-\), and with respect to \(R(\text{enz})\): \(\cdot OH > O_2- = H_2O_2 \gg e-\). The difference between the effectiveness of the most damaging and the least damaging...
free radicals was more than 10-fold greater in damage to enzyme than to membrane. Comparison between H2O2 added as a chemical reagent and H2O2 formed by irradiation showed that membranes and membrane-bound G3PDH were relatively inert to reagent H2O2 but markedly susceptible to the latter.
1. Introduction

Of all the free radicals produced in the radiolysis of water, e- and .OH have been the most extensively studied. Many reactions of hydrated electrons (e-) and hydroxyl radicals (.OH) with proteins and enzymes have been documented (Adams et al. 1969, 1971, 1973, Masuda, Ovadia and Grossweiner 1971, Lichtin, Ogdan and Stein 1972). Pulse radiolysis studies of such reactions have revealed certain amino acid residues which are particularly reactive towards these radicals. The rate constants of reactions of free radicals with biologically important molecules have been well documented by Anbar and Neta (1967). However, there are few studies of their interactions with biological membranes. Early studies of radiation effects on erythrocyte ghosts did not provide information on the reactions of free radicals with membranes (Goldstein 1974, Koter and Leyko 1973). Recent studies by Bisby et al. (1975) have attempted to investigate the reactions of e- and .OH with erythrocyte ghost membranes. They found that e- reacted rapidly with membranes at a rate greater than $10^4 \text{ M}^{-1}\text{s}^{-1}$. However, no reactions with the disulphide linkages of proteins were observed. This suggested the absence of
exposed disulphide bonds on the surfaces of the plasma membrane. The low reactivities of N-acetyl neuraminic acid and N-acetyl glucosamine which are on the exterior surface of erythrocyte membrane, cannot account for the observed reactivity of e-. Thus, the sites of reactions of e- remain unknown, one possibility being the peptide bonds of surface proteins. The carbonyl carbon of peptide linkage of proteins has been known to be quite reactive towards e- (Faraggi and Bettleheim 1977), but the possible reactions with peptide linkages were not investigated. Attack by .OH on aromatic amino acids of protein components of the membrane was not detected. However, transient species produced by the reactions of .OH with carbohydrate residues of the membrane surface were observed.

Barber et al. (1978) found that lecithin reacted efficiently with .OH and competed with other intramembrane solutes. This may explain the absence of products from the reactions between .OH and aromatic amino acids observed in Bisby's (1975) study. Also, reactions between .OH and solutes incorporated into the synthetic lipid bilayers indicated the freedom of diffusion of .OH through the bilayers. Consequences of .OH attack on lecithin bilayers
increased fluidity of the polar head group region, increased viscosity of hydrophobic region of the lecithin bilayers and appearance of water in the bilayers. In contrast, e- reacted very slowly with lecithin, the rate constant being about 10^7 M^-1.s^-1. The rate constants of the reactions of e- with solutes were decreased one thousand-fold when the solutes were solubilised in the lipid bilayers. The result indicated low reactivity of e- towards lipids and the restricted diffusion imposed on e- by the lipid layers.

Superoxide anions were reported to permeate stromal membranes of erythrocyte vesicles with surprising ease, and cause lysis of the membranes (Lynch and Fridovich 1978b) which is inhibited by superoxide dismutase. Lipid peroxidation caused by O_2^- is catalysed by metal complexes (Svingen 1978), and both O_2^- and singlet oxygen have been shown to propagate the resulting chain reactions. Singlet oxygen has been implicated in causing lysis of erythrocyte membranes (Bland et al 1978, Kelloq and Fridovich 1975, 1977, Lynch and Fridovich 1978b). A direct relationship between lipid peroxidation and lysis of erythrocyte ghosts has been demonstrated in our laboratory (Popma 1974, Davison and Popma 1978).
The reactions of free radicals with the SH-enzymes G3PDH and papain, have been studied (Buchanan and Armstrong 1978, Lin and Armstrong 1978). These studies suggest that oxidation of the SH groups at the catalytic sites were responsible for the inactivation of the enzymes. These SH groups, which are responsible for the covalent binding of acyl groups of the substrates possess marked reactivity towards electrophilic reagents (Friedman 1973). Thus, their reactions with oxidizing radicals would be much faster than those of less reactive SH groups. O2- was found to be one of the most effective radicals in causing irreversible inactivation on the enzyme, while H2C2 oxidized the SH groups to sulphenic acid causing damage which was reversed by DTT, thereby restoring the enzymic activity. Inactivation by the reducing radicals ,H and e- did not appear to be appreciable.

In a previous study (Kong 1979), resealed ghosts were shown to be suitable targets for studying the role of oxygen in free-radical damage in that they are sensitive to radiation damage and display a pronounced "oxygen effect", with oxygen enhancement ratios well over one. The present study is an
investigation of the effects of O$_2^-$, H$_2$O$_2$ and e$^-$ in producing damage to the plasma membranes of erythrocyte ghosts, as assessed by the change in permeability of the resealed ghosts and the inactivation of membrane-bound G3PDH. The inactivation of membrane-bound G3PDH provided information on the behaviour of these radicals with respect to an intracellular protein which is adsorbed on the membrane and an SH-enzyme of erythrocytes. Ghosts were subjected to the action of these free radicals, to assess their relative effectiveness in inducing damage to membranes. After initial experiments using chemical sources of free radicals, Gamma-irradiated aqueous solution was chosen as the source of these free radicals, which are formed from the radiolysis of water. Radiolysis of water in the absence of O$_2$ produces the following primary free radicals (Draganic and Draganic 1971, Swallow 1973).

$$\text{H}_2\text{O} \rightarrow e^-, H^+, \cdot \text{OH}, \text{H}_2\text{O}_2$$

and their initial yields are: $G(e^-)=2.8$, $G(H^+)=0.6$, $G(\cdot \text{OH})=2.8$ and $G(\text{H}_2\text{O}_2)=0.6$ (Draganic et al. 1969a, 1969b, 1971, 1972, 1973). $G$ is the number of molecules or ions formed per 100 ev. energy absorbed.
In the presence of oxygen, the following subsequent reactions occur, resulting in a variety of secondary radicals,

\[ \text{O}_2 + e^- \rightarrow \text{O}_2^- \quad k = 1.88 \times 10^{10} \text{ M}^{-1} \text{s}^{-1} \quad (2) \]

\[ \text{O}_2 + \text{H} \rightarrow \text{HO}_2 \quad k = 1.90 \times 10^{10} \text{ M}^{-1} \text{s}^{-1} \quad (3) \]

\[ \text{HO}_2 \rightarrow \text{H}^+ + \text{O}_2^- \quad pK = 4.8 \quad \text{(Behar 1970)} \quad (4) \]

\[ \text{HCOO}^- + \cdot \text{OH} \rightarrow \text{COO}^- + \text{H}_2\text{O} \quad k = 2.5 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \quad (5) \]

\[ \text{COO}^- + \text{O}_2 \rightarrow \text{CO}_2 + \text{O}_2^- \quad k = 2.4 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \quad (6) \]

The above rate constants were taken from Anbar and Neita (1967).

Some of these radicals can be selectively isolated from the mixture by using appropriate radical scavengers to remove other radicals. Thus, the effects of a single type of radicals or a combination of radicals can be studied independently. The further question of the results of interactions between these free radical forms of oxygen,
whether cooperative or inhibitory have also been investigated and the results will be presented in a subsequent publication (Kong 1979).
2. Experimental

2.1 Materials

Glyceraldehyde-3-phosphate (G3P), dithiothreitol (DTT), catalase, Triton X-100 and beta-NAD were supplied by Sigma Chemicals Ltd. Sodium arsenate, sodium pyrophosphate, sodium formate of A.C.S. standard were supplied by Fisher Chemicals Ltd. Superoxide dismutase was prepared in our laboratory according to the method of McCord and Fridovich (1969). The preparation has an activity of 2301 units/mg protein. Catalase was obtained from Sigma Chemicals with an activity of 2500 units/mg of protein. Both enzymes were added to a final concentration of 20 μg/ml (i.e. 20.4 units superoxide dismutase and 50 units catalase per ml solution) in the buffer solutions. These concentrations were confirmed to be sufficient to scavenge substantially in excess of 90% of the superoxide anions and H2O2 generated during irradiation (Sutton, Roberts and Winterbourn 1976, Davison and Kaminsky 1974).

2.2. Methods

See section 2.
2.3.1. Production of superoxide radicals

e- and \( \cdot \text{H} \) produced in (1) react with \( \text{O}_2 \) readily, and are converted to \( \text{O}_2^- \) by the reactions (2) and (3). Hence the irradiation of air-saturated solution will produce an amount of \( \text{O}_2^- \) equal to that of e- and \( \cdot \text{H} \). Catalase was used as scavenger for \( \text{H}_2\text{O}_2 \). It catalyses the decomposition of \( \text{H}_2\text{O}_2 \) as in (7). Formate is an effective scavenger of \( \cdot \text{OH} \) (Draganic 1969b) and it is used in our study because of the increase in yield of \( \text{O}_2^- \) due to reaction (6). Thus, irradiation of air-saturated aqueous solution containing 10 millimolar formate and catalase (50 units/ml) will produce predominantly \( \text{O}_2^- \). The total yield of \( \text{O}_2^- \) will be given by

\[
G(\text{O}_2^-) = G(\text{e}^-) + G(\cdot \text{H}) + G(\cdot \text{OH}) = 6.2
\]

\[
\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \longrightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad k = 3.4 \times 10^{7} \text{ M}^{-1} \text{s}^{-1} (7)
\]

(Herbert and Pinsent 1948)
2.3.2. Production of H2O2

If superoxide dismutase (20.4 units/ml) was used instead of catalase in the above solution, O2- produced will be converted to H2O2 according to (8).

\[ 2H^+ + O_2^- + O_2^- \longrightarrow H_2O_2 + O_2 \quad k = 2 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \quad (8) \]

(Rabani, Klug and Fridovich 1972)

Therefore \( G(H_2O_2) = G(H_2O_2) + 0.5xG(O_2^-) = 0.6 + 3.1 = 3.7 \)

2.3.3. Production of hydrated e-

In nitrogen saturated solution, the radicals formed are given in (1). It follows from the above discussion that formate in the irradiated solution will remove OH as well as H. Formate is not reactive towards e- \((k(HCOO^- + e^-) < 10^{-6})\) but very reactive towards H.

\[ H + HCOO^- \longrightarrow H_2 + COO^- \quad k = 2.5 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \quad (9) \]
Reaction (6) will not proceed in the absence of C2. Therefore, carboxyl radicals will be produced from reactions (5) and (9). The major radicals produced anaerobically are $e^- (G=2.8)$, CO$_2^-$ (G=3.4) and H$_2$O$_2$ (G=0.6).

2.3.4. Production of $\cdot$OH

$\cdot$OH radicals can be isolated in air-saturated buffer by adding both catalase and dismutase to the medium.
3. Results

3.1. Calculation of \( R(\text{mb}) \) and \( R(\text{enz}) \)

Figure 1 is a graph of the permeability of membranes as a function of time. The slopes of the curves in Figure 1 represent the rate at which \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \( \text{e}^- \) plus \( \text{CO}_2^- \), respectively, cause damage to the membrane. In order to compare the effectiveness of each kind of radical in causing the damage, each slope is divided by the yield \( G \) of the corresponding kind of radical per 100 ev. The ratio was denoted by \( R(\text{mb}) \). Similarly, \( R(\text{enz}) \) is calculated for each kind of radical. The \( R \) values are listed in Table 2. \( R(\text{mb}) \) and \( R(\text{enz}) \) were calculated assuming that all of the radicals formed contributed to the observed damage. This is of course not likely since in addition to disproportionation of the free radicals, a certain fraction of the radicals reacts with the membrane to produce damage to the membrane, while another fraction reacts with membrane-bound G3PDH, causing the inactivation. Assuming \( f \) to be the fraction of radical that reacts with the membrane; \( D \) be the observed rate of damage to membrane; \( r \) and \( R \) be the real and apparent value for rates of damage per radical, then
G . f . r = D

(10)

f . r = D/G

(11)

Since R = D/G

(12)

Therefore R = f . r

The same relation can be obtained for damage to membrane-bound G3PDH. Since we cannot estimate f, the true r values could not be obtained. Hence the R values calculated in our study were apparent values. f is proportional to the reactivity of the radical and r is proportional to the probability that one hit of the radical would produce damage, i.e., effectiveness of the radical. Thus, R represents the product of these two characteristics or the effective destructive power of the radical.

Since H2O2 was produced together with e- and CO2- in the irradiated formate solution saturated with nitrogen, the R values calculated may be affected by H2O2. The high yield
of unreactive CO₂⁻ (Adams, Redpath, Bisby and Cundall 1972) may cause a certain fraction of H₂O₂ to be reduced. The probability of e⁻ reacting with H₂O₂ would be very low owing to the high reactivity of e⁻ with the membrane. The removal of H₂O₂ would raise the R values of the reducing radicals. If 100% of H₂O₂ was removed by CO₂⁻, then both G(CO₂⁻) and G(e⁻) would be 2.8. R(mb) would then be 0.97x10⁻³. This would represent the maximum R(mb). On the other extreme, if there were no interactions between the radicals, i.e. damage by individual radicals were additive, then R(mb) would be 0.79x10⁻³. Hence the actual R(mb) for the reducing radicals would be

0.97x10⁻³ > R(mb) > 0.79x10⁻³

Similarly, the upper and lower bounds for R(enz) can be shown to be

1.14x10⁻³ > R(enz) > 0.25x10⁻³

H₂O₂ or O₂⁻ is produced as the only free radical in the air-saturated aqueous solutions containing formate and superoxide dismutase, or formate and catalase respectively.
However, oxygen present at 230 micromolar in the air-saturated solutions may further enhance any damage produced by these radicals, i.e., it may increase their R values. Unfortunately, such oxygen effects cannot be distinguished in our study, but if they were present they might be expected to show up in an autocatalytic character of the kinetics of destruction. Such effects were noted only for mixtures of radicals in the presence of 100% oxygen.

3.2. Effects of free radicals on activity of membrane-bound
G3PDH and on permeability of membranes

The logarithms of the percentage of intact ghosts remaining after a period of irradiation are plotted in Figure 1 which shows the effects of combinations of scavengers on membrane permeability, each combination chosen to yield selectively O2-, H2O2, ·OH or e- (Table 1). Similarly the loss of activity of membrane-bound G3PDH, expressed as a percentage of activity at zero dose, is plotted against time in Figure 2. In both figures, unirradiated ghosts were used as a control.
Table 1 summarizes the yields of free radicals produced under different conditions and the gradients of the corresponding curves plotted in Figures 1 and 2. The R values for individual radicals are compared in Table 2.

As shown in Figures 1 and 2, the logarithms of loss of enzymic activity and of increase in permeability of intact ghosts were linear with dose (or time) (the average correlation coefficient is 0.85), except for the increase of permeability due to \( \cdot \text{OH} \) (i.e. air-saturated solution containing both catalase and superoxide dismutase). In general, the rates of damage to membrane-bound G3PDH were about ten-fold greater than to permeability. In other words, the membrane permeability is more resistant to damage by radicals than is membrane-bound G3PDH. Irradiation of air-saturated solutions containing formate in the presence of either catalase or superoxide dismutase, produced the same change in permeability but different rates of inactivation of G3PDH. Anaerobic solutions provided significant protection to membrane-bound G3PDH. Comparison of the rates obtained from the slopes of the graphs do not directly indicate the effectiveness of each kind of radical in inducing damage, due to the different quantities of
radicals produced under the different conditions. The R
values in Table 2 offer a more accurate comparison of these
radicals. \( \cdot \text{OH}, \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) were about equally effective in
inactivating membrane-bound G3PDH while \( e^- \) and \( \text{CC}_2^- \) were the
least effective. \( R(\text{enz}) \) for \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) were 12-fold and
for \( \cdot \text{OH} \) was 17-fold that of \( e^- \). \( R(\text{mb}) \) of \( e^- \) and \( \text{H}_2\text{O}_2 \) were
about 1.5-fold and of \( \cdot \text{OH} \) 3-fold of that of \( \text{O}_2^- \). Therefore,
the difference between the effectiveness of the most
damaging and the least damaging free radicals was greater in
damage to enzyme than to membrane; at least 10 times the
latter. Hence, in the order of decreasing \( R(\text{mb}) \): \( \cdot \text{OH} > e^- = \text{H}_2\text{O}_2 > \text{O}_2^- \) and in order of decreasing \( R(\text{enz}) \): \( \cdot \text{OH} >> \text{O}_2^- = \text{H}_2\text{O}_2 >> e^- \). Thus \( \cdot \text{OH} \) was the most powerful radical in
causing damage to both membrane and membrane-bound G3PDH.

3.3. Comparison of reagent \( \text{H}_2\text{O}_2 \) with \( \text{H}_2\text{O}_2 \) produced by
radiation

Reagent \( \text{H}_2\text{O}_2 \) was added to a ghost suspension to a final
concentration of 120 micromolar. Comparison of the damage
due to reagent \( \text{H}_2\text{O}_2 \) with that due to \( \text{H}_2\text{O}_2 \) produced by
irradiation in the presence of formate plus superoxide
dismutase indicated a striking difference (Figures 3 and 4).
Even though no R value can be calculated for reagent H2O2, it is obvious that radiolytically produced H2O2 is far more destructive.
4. Discussion.

Each of the radicals, .OH, H2O2, O2- and e-(plus CO2-) produced in radiolysis of water was more destructive to membrane-bound G3PDH than to the membrane permeability by an order of magnitude, despite the relative inaccessibility of G3PDH. This striking difference may be attributed largely to the hydrophilic character of these radicals which, when combined with the lack of water inside the lipid bilayers, effectively excludes the production of these damaging radicals in the midst of vulnerable lipids, since sites of unsaturated fatty acids are known to occur deep within the hydrophobic region of the membrane. Alternatively, several neighbouring sites of damage may be required to produce an observable increase in permeability.

The greater inactivation of G3PDH by radicals with high reduction potentials indicates that oxidative damage may be the major means of inactivation. This supports the findings that the oxidation of the sulfhydryl group at the enzyme catalytic site was found to be related to the inactivation of the enzyme (Buchanan and Armstrong 1978, Lin and Armstrong 1978). The linear relationship between the
logarithm of enzyme activity and dose, as found in most
irradiated enzymes (Swallow 1973), indicates that a constant
fraction of enzyme was inactivated by a given amount of
radiation. This behaviour was expected since the
probability of the radicals reacting with the active and
inactivated enzyme should be similar.

Since formate is the only scavenger among those used that
can diffuse through the membrane of erythrocytes (Whittam
1964), superoxide dismutase and catalase can only remove O2-
and H2O2 formed on the outside or that diffusing to the
compartment of resealed ghosts. However, the quantity of free
radicals formed on the outside of the ghosts is
significantly greater than that inside the ghosts owing to
the much smaller volume of the ghosts compared to the bulk
of the solution. Moreover, the ghost membrane is permeable
to H2O2 (established in our experiments) and O2- (Lynch and
Fridovich 1978a), hence these radicals can establish an
equilibrium concentration on both sides of the membrane.
Thus, the effects of these two scavengers will be less
affected by the permeability barrier of the ghosts than
might at first be expected. This is supported by findings
that externally added superoxide dismutase can effectively
inhibits lysis of erythrocyte vesicles by O$_2^-$ generated inside the vesicles (Lynch and Fridovich 1978b).

Furthermore, residual catalase and dismutase in the ghosts would remove most of the free radicals formed in the cytoplasmic space of the ghosts.

4.1. Effects of superoxide anions

In comparison with the other free radicals studied, O$_2^-$ is the least effective in causing lysis of the ghost membranes. A much more effective damage to erythrocyte vesicles by O$_2^-$ was reported by Lynch (1978b). However, the presence of complexed metals in their reaction medium may have catalysed the lipid peroxidation by O$_2^-$ (Svingen 1978) as well as by .OH formed in the xanthine-xanthine oxidase system (Buettner 1978). The mechanism by which O$_2^-$ increases permeability is not established in the present study. It may be that O$_2^-$ causes oxidation of lipids in membrane by reacting with their unsaturated fatty acids. Although its reactivity towards olefines is low (Primer and Rosenthal 1978, Bielski and Richter 1977, Kaschnitz and Hatefi 1975), complexed metals such as adenosine diphosphate metal complexes are known to catalyse lipid peroxidation by O$_2^-$ (Svingen 1978).
In view of the residual content of resealed ghosts, reaction of $O_2^-$ with double bonds of lipids may be possible. A more plausible mechanism is the reaction of $O_2^-$ with membrane SH groups, since it is known to be reactive towards SH groups (discussed below). The observations that lipid peroxidation increases the content of reactive SH groups in the membrane, whereas agents binding SH groups accelerate the onset of lipid peroxidation (Robinson 1965, 1966), suggest that $O_2^-$ might increase permeability by oxidizing the membrane SH groups. That the reduced state of the membrane SH groups is vital to the structural integrity of membrane is further demonstrated by the lysis of membranes as a result of the reaction of SH groups with SH agents (Carter 1973, Zipursky, Stephens, Brown and Larsen 1974). Some of these SH groups are shown to be accessible to water soluble agents (Zipursky, Stephens, Brown and Larsen 1974). The low $R_{mb}$ may be due to the negative charge of $O_2^-$. The charge on this radical would have hindered its diffusion into the hydrophobic region of the plasma membrane, thus preventing it from reacting with the unsaturated bonds of the fatty acids in the lipid layers. Its reaction with the components of the membrane would thus be confined to those exposed on the surface of the membrane of ghosts. Its exclusion from
the more reactive lipid region of the membrane probably prevented the radical from producing a significant amount of damage to the membrane's permeability.

In contrast, O2- was one of the most strongly inactivating radicals towards membrane-bound G3PDH. This contradictory behaviour of O2- can be explained by its reactivity towards SH groups. It was shown that O2- was reactive towards SH groups in the active sites of pure G3PDH i.e. the SH groups belonging to cys 149 in the four subunits (Conway and Koshland 1968, Teipel and Koshland 1970). The low pK of 5.4 observed in the holoenzyme (Buchanan and Armstrong 1978) suggested that ionisation of SH probably facilitates the initial damage to G3PDH by O2- in reaction (10). Lin (1978) suggested the sequence of reactions (13)-(18) to account for the inactivation of SH-enzymes by O2-.

\[
\text{E-SH} + H^+ + O2^- \rightarrow \text{E-S} + H_2O_2 \quad (13)
\]

\[
\text{E-S} + O2 \rightarrow \text{E-SOO} \quad (14)
\]

\[
\text{E-SOO} + O2^- + H^+ \rightarrow \text{E-SOOH} + O2 \quad (15)
\]
E-SOOH + H2O $\rightarrow$ E-SOH + H2O2 \hspace{1cm} (16)

E-SOH + O2- + H+ $\rightarrow$ E-SO. + H2O2 \hspace{1cm} (17)

E-SO. $\rightarrow$ inactive enzyme \hspace{1cm} (18)

In spite of the facts that the amount of O2- produced in the bulk of the medium is significantly greater than that produced inside the ghosts, that it is hydrophilic, and that residual superoxide dismutase is present in the ghosts, O2- is effective in inactivating G3PDH for three reasons.

Firstly, resealed ghosts are permeable to O2- (Lynch and Fridovich 1978a). Thus O2- produced outside ghosts can react with G3PDH on the cytoplasmic side. This permeability of membranes to O2- implies existence of anion channels because the negative charge on O2- would otherwise hinder its diffusion through the lipid bilayers. Secondly, its low reactivity towards membranes allowed most of O2- to permeate the membrane and react with G3PDH. Other membranous SH groups, including those of residual hemoglobin in the ghosts are probably secondary sites of attack by O2-. This is due to the lower reactivity of intramembranous SH than the aqueous catalytic SH groups. Thirdly, the residual
superoxide dismutase in our ghosts removes some of O2- on the cytoplasmic side of the membrane. However, C2- reaching an element of volume adjacent to the cytoplasmic side of the membrane would not be effectively removed by the residual enzyme due to the electrostatic repulsion between the membrane and dismutase because most of the enzyme would be negatively charged (Lynch and Fridovich 1978b), its isoelectric point being 4.95 (Bannister, Bannister and Wood 1971). This would mean a diminished concentration of dismutase in the element of volume adjacent to the internal surface of the membrane where G3PDH is located. Therefore the small amount of residual dismutase would not affect the R(mb) or R(enz) of O2- significantly.

4.2. Effects of hydrated electrons

From our data, we cannot separate the effects of e- and CO2- since they were produced together. However, most of the damage in the presence of N2 plus formate can be attributed to e- in view of the relatively low reactivity of the CO2- (Adams, Redpath, Bisby and Cundall 1972), and the relatively small amounts of H2O2 produced (Table 1). The sphere of water around the hydrated e- impedes its freedom to traverse
the lipid bilayers. Even if it can pass through anion channels like O2-, its relatively higher reactivity would cause most of it to be consumed in reactions within the channels. In solution therefore, one would expect its access to reactions with proteins bound on the cytoplasmic side or within the membrane to be limited. The concentration of e- produced on the cytoplasmic side is only a small fraction of the total radical yield. Also, owing to the high reactivity of e- with the surface of the membrane, most of e- is removed by the membrane. These considerations may explain the low level of inactivation of membrane-bound G3PDH. Results from Bisby (1978) and Barber (1978) seem to support this explanation. Barber found a thousand fold drop in the rate constants of the reactions of e- due to the lipid-layer and Bisby confirmed the low reactivity towards membranous SH groups of erythrocyte ghosts.

The greater damage to membrane permeability caused by e- in comparison with O2- is probably due to its higher reactivity. Nevertheless, the moderate damage again reflects the restricted reactivity of e- with lipids due to its hydrophilic character. Carbonyl carbon of the peptide bonds in surface proteins probably comprise one of the
target sites. The main consequence of this interaction is reductive deamination (Neta, Simić and Hayon 1970, Paraggi and Battleheim 1977, Plossmann and Westhof 1978, Mittal and Hayon 1974, Rustgi, Joshi and Riesz 1977) leading to peptide breakage and release of malonaldehydes (Tappel and Adhikari 1975).

\[-\text{NH-CHR-C}=\text{O} + \text{e}^- \rightarrow \text{NH-CHR-}\overset{\cdot}{\text{C}}=\text{O}^-\]  \hfill (19)

\[-\text{NH-CHR-}\overset{\cdot}{\text{C}}=\text{O}^- + \text{H}^+ \rightarrow \text{NH}_2 + \text{CHR}=\text{C}=\text{O}^-\]  \hfill (20)

\[\text{CHR}=\text{C}=\text{O}^- \rightarrow \overset{\cdot}{\text{CHR}}-\text{C}=\text{O}\]  \hfill (21)

4.3. Effects of H2O2

H2O2 was found to cause reversible damage to pure G3PDH and to papain (Buchanan and Armstrong 1978, Lin and Armstrong 1978) by oxidizing the enzymic SH to sulphenic acid which can be reduced to SH by DTT.

\[\text{E-SH} + \text{H}_2\text{O}_2 \rightarrow \text{E-SOH} + \text{H}_2\text{O}\]  \hfill (22)
However, our data revealed only the additional irreversible inactivation because incubation with DTT did not restore enzyme activity. Reversible damage, if present, would not have been detected in the current study since DTT was routinely present in the assay medium, the possible contribution of \( \cdot \text{OH} \) and \( \text{O}_2^- \) in the presence of both formate and superoxide dismutase is negligible due to the effectiveness of these scavengers. Singlet \( \text{O}_2 \) may however be involved as it is thermodynamically feasible to produce singlet \( \text{O}_2 \) via \( \text{O}_2^- \)-dismutation. However, this is not likely when the dismutation was catalysed by superoxide dismutase (Koppenol and Butler 1977, Petkau and Chelack 1976).

Furthermore, the residual catalase found in our ghosts is 12% of that of red blood cells, and unlike superoxide dismutase, has a higher isoelectric point (pH 5.7). This means that the residual catalase is more effective in protecting membrane-bound G3PDH. Thus the observed damage to G3PDH requires some other explanation. Very likely, some reactive species, perhaps lipid peroxides, arising from the

\[
\begin{align*}
E-\text{SOH} + \text{HS-D-SH} & \rightarrow E-\text{SS-D-SH} + \text{H}_2\text{O} \quad (23) \\
E-\text{SS-D-SH} & \rightarrow E-\text{SH} + \text{DS2} \quad (24)
\end{align*}
\]
interaction of H2O2 with the membranes could not be removed by catalase. Catalase added externally protects both membrane and membrane-bound G3PDH (more detailed study has been submitted for publication) thus indicating that external catalase effectively removed H2O2 produced in the bulk of the aqueous medium. The R(mb) and R(enz) of H2O2 are the sums of damage due to H2O2 and the reactive species.

The difference in reactivity towards cellular constituents between reagent H2O2, and H2O2 produced by radiation was observed by Klebanoff (1958). It was found that much higher concentrations of reagent H2O2 had to be used to produce effects equal in magnitude to the effects of radiation. Alder (1963) found that H2O2 which had been generated enzymically, produced damage to cells comparable to that of H2O2 produced by radiation. This suggests that a continuous low level of H2O2 is more damaging than a maximum dose, or that free radicals mediating or accompanying its production were responsible for most of the damage.
4.4. Effects of .OH

The high \( R(\text{mb}) \) for .OH could be explained by the high reactivity of .OH towards phosphatidyl choline (rate constant is \( 5.1 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \)) (Barber and Thomas 1978), and the lack of restriction by the hydrophobic region of the membranes upon its movement. Since erythrocyte membranes contain approximately 30 mole% of phosphatidyl choline, they are particularly susceptible to attack by .OH. The crosslinking between lipids and the increase in fluidity of the polar head region observed in lecithin bilayers attacked by .OH may also occur in ghost membranes, leading to penetration of water channels into the hydrophobic region of the membrane. Also, cholesterol which exists in equimolar concentration to phospholipids in the erythrocyte membrane, is known to form hydroperoxides with .OH (Allen, A.O. 1961). The hydroperoxides disrupt packing of lipids in the membrane, leading to leakiness of the membrane (Lamola, Yamane and Trozzolo 1973, Bland, Madden and Herbert 1975), in addition to triggering autocatalytic lipid autoxidation. The acceleration of the damage to membranes in the presence of oxygen observed may be due to free radical chain reactions occurring in the hydrophobic region where the
unsaturated fatty acids predominate. The organic radicals formed by the addition of .OH or abstraction of .H from organic molecules by .OH, are mainly consumed by addition reactions or by disproportionation. However, in the presence of O2, they react at diffusion controlled rates with O2 to produce O2- or hydroperoxides. The presence of water channels in the hydrophobic region as a result of damage together with water formed in reaction (25) further facilitates the production and mobility of O2-. Thus O2- radicals probably aid in propagating the chain reactions within the membrane, and in this location are unlikely to be scavenged by superoxide dismutase outside the membranes. Radiolysis of water previously excluded from the midzone of membranes due to the hydrophobic nature of this region could then occur, and so accelerates damage to membranes. The curvature of the plot of damage to membranes in the presence of catalase and dismutase could be partly due to this effect (Figure 1). The water channels observed by Barber (1978) may be partly due to this reaction.

\[ \text{RH} + .\text{OH} \rightarrow \text{R} + \text{H}_2\text{O} \]  
(25)
The common feature of O₂-, H₂O₂ and .OH that enables them to produce damage to membrane-bound G3PDH is the permeability of the membranes to these radicals. The higher R(enz) for .OH was attributed to its higher reduction potential. Bray (1970) reported a value of +2.3 V. for .OH/H₂O pair. This value compared to +1.1 and +0.4 V. for O₂- and H₂O₂ respectively (George 1965, Mason 1965). This is in agreement with the order of R(enz) of these radicals.

The factors governing the effectiveness of a free radical in causing damage to membrane permeability and membrane-bound G3PDH are many and complex. However, our study taken with the results of others, allows us to define some of these factors: (a) the reactivity of the radical towards specific components of membrane (or components of membrane-bound G3PDH) the alteration of which would effectively affect the integrity of the membrane (or membrane-bound G3PDH); for instance, the effectiveness of the oxidative free radicals agree with their known reactivity towards SH groups at the active site of membrane-bound G3PDH and the reactivity of .OH towards lecithin of membrane. (b) Accessibility of such components to the radical. This includes permeability characteristics of the membrane to the radical as well as
the extent of exposure of the reactive groups on the membrane. The normal permeability of the ghost membrane allowed O₂- to attack SH groups on the cytoplasmic side of the membrane, while e-, though more reactive, was diminished in effectiveness due to the restricted diffusion imposed on it by the lipid layers. Damage to the membrane can also alter the accessibility of its susceptible sites to free radicals. Conformational changes of damaged membrane proteins may produce deleterious effects on packing of lipid bilayers (Yonei, Todo and Kato 1979), and such changes also render the SH groups and aromatic residues in the intramembranous portion of the proteins more accessible to radical attack. Lipids associated with the proteins also become more accessible to free radicals by virtue of the aqueous channels provided by the conformational changes of the membranous proteins as well as the decreased hydrophobic interactions of protein SH groups with the lipids (Robinson 1965, 1966, Carter 1973). (c) Reactions of the radical with other components whose alteration produced only minor effects. This is an important factor in determining the quantities of radical reaching the components mentioned in (a). These components could be considered intrinsic nonspecific scavengers in the membrane. The surprisingly
Moderate damage by e- to membranes as compared to its high reactivity illustrates this. Most of the e- which attempts to diffuse through the membrane via aqueous channels formed by proteins reacts with these proteins and very little thus reaches the cytoplasmic side. (d) Hydrophilic character of the radical. All four radicals studied are either charged or polar. The highly unfavourable entropic factor in interactions of these radicals with the lipids is probably one of the most important determinants of the relatively low damage to the membranes caused by these radicals.

We conclude that the interaction of free radicals with the membrane caused destruction of membrane proteins and lipid, resulting in an increase in permeability. The fact that the damage due to four different kinds of radicals, cf different redox potentials and reactivities, were of a similar order of magnitude demonstrates that a wide variety of reactions contributes effectively to the breakdown of the permeability barrier. Furthermore, the proteins associated with the membranes were protected against free radicals by the lipids of the membrane, except in the case of those radicals to which the membrane was permeable. These factors in the design of lipid bilayers in the plasma membrane thus appears to have been of evolutionary advantage.
TABLE 1. EFFECTS OF FREE RADICALS ON PERMEABILITY AND ACTIVITY OF MEMBRANE-BOUND G3PDH. The yields of free radicals were given in molecules or ions per 100 ev. of radiation. The rate of damage to permeability and activity of G3PDH were calculated from the slopes of plots given in this paper. The rates were expressed in log per cent of ghosts remaining intact and log per cent activity remaining after each hour of irradiation. Conditions were: 0.01 M. phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mosM., temperature 25 deg C, concentration of ghosts or erythrocytes 10% v/v, dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffer solutions saturated by atmospheric air unless oxygen or nitrogen is specified. Catalase, superoxide dismutase or formate when present were at final concentrations of 50 units/ml, 20.4 units/ml, and 10 mM. in the buffer solutions. The slopes and their standard errors were obtained by regression analysis (see Methods). The standard errors of most of the slopes of the plots of permeability and enzymic inactivation are less than +/-30% and +/-20% respectively.
<table>
<thead>
<tr>
<th>scavengers</th>
<th>radicals produced</th>
<th>rate of increase</th>
<th>inactivation of G3PDH permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>formate</td>
<td>3.7</td>
<td>3.5 +/- 1.6</td>
<td>29.74 +/- 3.9</td>
</tr>
<tr>
<td>dismutase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>formate</td>
<td>6.2</td>
<td>3.93 +/- 1.07</td>
<td>53.93 +/- 8.76</td>
</tr>
<tr>
<td>catalase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>0.6</td>
<td>2.8</td>
<td>3.4 5.45 +/- 1.33 6.40 +/- 2.27</td>
</tr>
<tr>
<td>formate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>catalase</td>
<td>2.8</td>
<td>5.05 +/- 1.42</td>
<td>32.47 +/- 3.68</td>
</tr>
<tr>
<td>dismutase</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2. THE EFFECTIVENESS OF H₂O₂, O₂⁻, e⁻ AND .OH IN CAUSING DAMAGE TO PLASMA MEMBRANE AND MEMBRANE-BOUND G3PDH.

\( R(\text{mb}) \) and \( R(\text{enz}) \) were obtained by dividing the rates in Table 1 by the yield of the corresponding free radical in molecules or ions per 100 ev. Conditions were as described in Table 1.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>( R(\text{mb}) )</th>
<th>( R(\text{enz}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 µM H₂O₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 µM e⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 µM .OH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Radical</th>
<th>R(mB) x 10^-3</th>
<th>R(enz) x 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O2</td>
<td>0.95±0.47</td>
<td>8.04±1.38</td>
</tr>
<tr>
<td>O2^-</td>
<td>0.63±0.2</td>
<td>8.69±1.76</td>
</tr>
<tr>
<td>e^- + CO2^-</td>
<td>&gt;0.73±0.22</td>
<td>&gt;0.25±0.1</td>
</tr>
<tr>
<td></td>
<td>&lt;0.97±0.27</td>
<td>&lt;1.14±0.45</td>
</tr>
<tr>
<td>.OH</td>
<td>1.80±0.58</td>
<td>11.60±1.78</td>
</tr>
</tbody>
</table>
FIGURE 1. COMPARISON OF THE EFFECTS OF O$_2^-$, H$_2$O$_2$, .OH AND e- ON PERMEABILITY OF PLASMA MEMBRANES OF GHOSTS. Log percent ghosts remaining intact after each hour of irradiation were plotted against time in hour. Conditions were: 0.01 M. phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mosM., temperature 25 deg C, concentration of ghosts or erythrocytes 10%v/v, dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffer solutions saturated by atmospheric air unless oxygen or nitrogen is specified. Catalase, superoxide dismutase or formate when present were at final concentrations of 50 units/ml, 20.4 units/ml, and 10mm. in the buffer solutions. The slopes and their standard errors were obtained by regression analysis described in Methods.

Symbols

Control  
.OH  
O$_2^-$  
H$_2$O$_2$  
H$_2$O$_2$ + e- + CO$_2^-$
FIGURE 1. COMPARISON OF THE EFFECTS OF O₂⁻, H₂O₂, OH⁻ AND e⁻ ON PERMEABILITY OF PLASMA MEMBRANES OF GHOSTS.
FIGURE 2. COMPARISON OF THE EFFECTS OF O₂-, H₂O₂, ·OH AND e⁻ ON ACTIVITY OF MEMBRANE-BOUND G3PDH OF GHOSTS. Log percent activity remaining after each hour of irradiation were plotted against time in hour. Conditions were as described in Figure 1.

Symbols

- Control
- ·OH
- O₂⁻
- H₂O₂
- H₂O₂ + e⁻ + CO₂⁻
FIGURE 2. COMPARISON OF THE EFFECTS OF \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), \( \cdot \text{OH} \) AND \( e^- \) ON ACTIVITY OF MEMBRANE-BOUND G3PDH OF GHOSTS.
FIGURE 3. COMPARISON OF THE EFFECT OF REAGENT H2C2 AND RADIATION INDUCED H2C2 ON PERMEABILITY OF MEMBRANES. Log per cent ghosts remaining intact after each hour of irradiation were plotted against time in hour. Conditions were as described in Figure 1.

Symbols

- Control
- Radiation-induced H2O2
- Reagent H2O2
FIGURE 3. COMPARISON OF THE EFFECT OF REAGENT H$_2$O$_2$ AND RADIATION INDUCED H$_2$O$_2$ ON PERMEABILITY OF MEMBRANES.
FIGURE 4. COMPARISON OF THE EFFECT OF REAGENT H₂O₂ AND RADIATION INDUCED H₂O₂ ON ACTIVITY OF MEMBRANE-BOUND G3PDH.

Log percent activity remaining after each hour of irradiation were plotted against time in hour. Conditions were as described in Figure 1.

Symbols

- Control
- Radiation-induced H₂O₂
- Reagent H₂O₂
FIGURE 4. COMPARISON OF THE EFFECT OF REAGENT H2O2 AND RADIATION INDUCED H2O2 ON ACTIVITY OF MEMBRANE-BOUND G3PDH.
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THE ROLE OF INTERACTIONS BETWEEN O₂-, H₂O₂, •OH AND e⁻ AND OXYGEN ON THE PERMEABILITY OF MEMBRANES AND ACTIVITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF RESEALED GHOSTS.

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ABSTRACT

The occurrence of the Haber-Weiss reaction and other interactions between free radicals has been investigated in the effects of mixtures of free radicals on permeability of ghosts and on the activity of membrane-bound glyceraldehyde-3-phosphate dehydrogenase (G3PDH) of resealed erythrocyte ghosts. The following mixtures were found to induce damage greater than that which could be accounted for by the independent actions of the constituent free radicals: (1) \( \cdot \text{OH} + \text{H}_2\text{O}_2 \), and (2) \( \cdot \text{OH} + \text{H}_2\text{O}_2 + \text{O}_2^- \). In contrast, the following mixtures were found to induce less damage than that predicted on the basis of independent actions of constituent free radicals: (1) \( \text{H}_2\text{O}_2 + \text{O}_2^- \), and (2) oxidizing radicals \( (\cdot \text{OH}, \text{H}_2\text{O}_2) \) + reducing radicals \( (\text{e}^-, \text{H}^+) \).

These results suggest a Haber-Weiss like interaction between \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) and an interaction between \( \text{H}_2\text{O}_2 \) and \( \cdot \text{OH} \) to produce a species more potent than either in causing increased permeability. The decrease in damage observed in the simultaneous presence of oxidizing and reducing radicals suggested an antagonistic effect by which each tends to moderate damage by the other. Inactivation of G3PDH was found to be more sensitive to radiation than permeability by
an order of magnitude, while permeability was more sensitive to the enhancement of damage by oxygen. Comparison of the effectiveness of free radical scavengers in inhibiting the increase in permeability caused by free radicals showed the following order of effectiveness expressed in terms of percentage of protection:

Formate (90%) > nitrogen (65%) > catalase (60%) > dismutase (32%)

and with respect to enzymic inactivation,

nitrogen (100%) > formate (77%) > dismutase (48%) = catalase (44%)

The findings of the present study allow us to propose a mechanism for the "oxygen effect" in radiation damage. The "oxygen effect" is due to the interaction of oxygen with e- and H., producing O2-. As a result, a Haber-Weiss like reaction can occur. The further increase in damage at increased concentrations of oxygen is due to the interaction of oxygen with sites of initial damage.
1. Introduction.

Of the interactions between free radicals, two important examples widely encountered in studies of biological damage mediated by oxygen are: (1) the interaction between H2O2 and O2- and (2) an unexplained interaction between the concentration of oxygen and the extent of radiation damage, the so-called "oxygen effect". The effect of oxygen on radiation damage to cells has been well documented (Koch, Kruuv and Frey 1973, Howard-Flanders 1960, Ewing 1978, Van Hemmen, Meuling and Bleichrodt 1978, Yuhas and Li 1978). The effect of oxygen in enhancing cellular damage while invariably observed, is still unexplained (Alper 1956, Adams 1972). Attempts to elucidate the mechanism of the oxygen effect have been made in studies of radiation damage to cellular components such as sulphydryl compounds (Quintiliani, Badiello, Tamba, Esfandi and Gorin 1977, Purdie 1971), nucleotides (Michaels and Hunt 1977, Srivastava 1974, Van der Schans and Blok 1969), and lipids (Wills and Wilkinson 1967, Raleigh, Krembers and Gaboury 1977). Biological membranes have been considered to be critical targets in irradiated cells (Alper 1968) and to be major loci for the development of oxygen effect (Alper
In previous studies (Kong 1979, Davison and Kong 1977), we have shown that whereas the presence of oxygen usually confers protection against radiation damage to purified proteins in aqueous solution (Davison and Kaminsky 1974), the permeability and activity of membrane-bound glyceraldehyde-3-phosphate dehydrogenase (G3PDH) of resealed erythrocyte ghosts showed a marked enhancement in the presence of oxygen. Hence, resealed ghosts constitute one of a small number of radiation targets which can be used to investigate the "oxygen effect" in a relatively well characterized medium.

The reactions:

\[ \cdot \text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \cdot \text{O}_2. \]  \hspace{1cm} (1)

\[ \cdot \text{O}_2 + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{H}_2\text{O} + \text{O}_2 \]  \hspace{1cm} (2)

were proposed originally by Haber and Willstatter in 1931 as chain reactions in a mechanism for the decomposition of \( \text{H}_2\text{O}_2 \) initiated by iron ions. These reactions became later known as the "Haber-Weiss cycle" (Haber and Weiss 1934). Reaction (2) has frequently been invoked (Beauchamp and Fridovich
1970, Goscin and Fridovich 1972, Hodgson and Fridovich 1976a, Hodgson and Fridovich 1976b, McCord 1974, Fee and Teitelbaum 1972, Zimmermann, Flohe, Weser and Hartmann 1973) to explain the fact that catalase and dismutase protect biological systems against damage much more than the individual reactivities of $O_2^-$ or $H_2O_2$ would suggest. Since attempts to demonstrate reaction (2) have failed (Halliwell 1976, McClune and Fee 1976), and several authors have reported a small rate constant (less than 2 M$^{-1}$s$^{-1}$) for this reaction (Ferradini, Poos, Houee and Pucheault 1978, Kopp€nol, Butler and van Leeuwen 1978, Dainton and Rowbottom 1953, Czapski and Allan 1962), the validity of using reaction (2) to explain previous observations was therefore questioned. However, it was found that complexed metals catalyse reaction (2) (Fong, McCay, Poyer, Misra and Keele 1973, 1976, McCord and Day 1978, Halliwell 1978) in a "Fenton" type reaction. In this mechanism, $O_2^-$ reduces an iron (III) (or other transition metal) chelate. The reduced metal may then react with $H_2O_2$ to produce .OH, thus:

$$M(N)^+ + O_2^- \rightarrow M(N-1)^+ + O_2$$  \hspace{1cm} (3)

$$M(N-1)^+ + H_2O_2 \rightarrow M(N)^+ + .OH + OH^-$$  \hspace{1cm} (4)
In view of the abundance of metalloproteins and complexed metals in biological tissues, reactions (3) and (4) may well occur in processes causing damage to biological systems.

In some systems, a protective role for O2- has been inferred from a destructive action of superoxide dismutase in radiation induced damage to cytochrome c (Davison and Kaminsky 1974) and from a decreased production of ethylene in the reaction of 6-hydroxydopamine with oxygen (Cohen and Heikkela 1974). In contrast, H2O2 was found to enhance damage to bacterial spores caused by .OH (Powers 1972).

The study of the complex interactions between free radicals is thus prerequisite to a better understanding of the behaviour of mixtures of free radicals in, and their toxic effects on, biological systems. The technique employed in our previous study (Kong 1979) for isolating individual free radicals proved to be a useful tool for production of well characterized and relatively clean mixtures of free radicals. Making use of the knowledge of kinetics of damage to ghosts by different free radicals (Kong 1979), we will show the occurrence of the Haber-Weiss reaction and other
interactions between free radicals including oxygen. We will also consider the implications of these findings for selecting among various possible mechanisms for the "oxygen effect".
2. Experimental

2.1 Materials

Glyceraldehyde-3-phosphate (G3P), dithiothreitol (DTT), catalase, Triton X-100 and beta-NAD were supplied by Sigma Chemicals Ltd. Sodium arsenate, sodium pyrophosphate, sodium formate of A.C.S. standard were supplied by Fisher Chemicals Ltd. Superoxide dismutase was prepared in our laboratory according to the method of McCord and Fridovich (1969). The preparation has an activity of 2301 units/mg protein. Catalase was obtained from Sigma Chemicals with an activity of 2500 units/mg of protein. Both enzymes were added to a final concentration of 20 μg/ml (i.e. 20.4 units superoxide dismutase and 50 units catalase per ml solution) in the buffer solutions, these concentrations are sufficient to scavenge virtually all the superoxide anions and H2O2 generated during irradiation (Sutton, Roberts and Winterbourn 1976, Davison and Kaminsky 1974).

2.2. Methods

See section 2.
2.2.1. Analysis of data

Each of the slopes of the graphs in Figures 1 to 6 were obtained by regression analysis of 18 experimental observations representing on the average 3 separate experiments. The regression analysis was carried out by linear regression using the University of Alberta APL statistical package "STP2". The results, including comparison of initial and final slopes, were further confirmed using the APL program "ANCOVA1" based on the methods described in Sckal and Rohlf (1969) for analysis of covariance. The standard errors of the slopes of graphs of permeability as a function of time are on the average less than +/-30% while those of enzymic inactivation are less than +/-20%. The ratios in Tables 3 and 4 are analysed by t test for deviations from unity. Details are discussed in the descriptions of the corresponding tables. Standard errors of radiochemical yields (G) are of the order of 4% (Draganic et al. 1969a, 1969b, 1971, 1973). The asymmetry of the gamma source, the inevitable variation in the cytoplasmic contents and tocopherol content of the membrane preparations are probably the major sources of errors that
account for the standard deviations of our data. However, these effects would be small in comparison to the effects of free radicals since irradiation in the absence of externally added scavengers produced significant damage to the membranes.
3. Results.

Figures 1 to 6 represent plots of permeability and activity of G3PDH as a function of irradiation dosage (time) in the presence of different combinations of scavengers. Tables 1 and 2 summarize the slopes of these plots together with the yields of free radicals under the stated conditions. These slopes represent the rates of damage to the membrane and to G3PDH by the various mixtures of free radicals. From the \( R(\text{mb}) \) and \( R(\text{enz}) \) values for the individual free radicals established in a previous study (Kong 1979), the theoretical rates of damage by each mixture of free radicals can be calculated. These calculated values are to be compared to the slopes of the plots in the last columns in tables 3 and 4. Ratios much greater than or less than 1 indicates disagreement between the calculated and experimental rates, and therefore synergistic cooperation between, or mutual annihilation of, the free radicals causing the biological damage. The initial and final slopes of the plots of permeability and enzymic inactivation in aerated, anaerobic and oxygenated buffers are compared in Tables 5 and 6. These ratios express the oxygen enhancement of permeability and enzymic inactivation.
Cooperation in inducing damage was noted among constituents of the following mixtures of free radicals: (1) $\text{H}_2\text{O}_2 + .\text{OH} + \text{O}_2^-$, (2) $.\text{OH} + \text{H}_2\text{O}_2$, and interactions decreasing damage were noted in (3) $\text{H}_2\text{O}_2 + \text{O}_2^-$ in the presence of formate, (4) oxidizing radicals ($\text{.OH} + \text{H}_2\text{O}_2$) + reducing radicals (e$^- + \text{H}_2$).

3.1. Mixtures of $\text{.OH}$, $\text{H}_2\text{O}_2$ and $\text{O}_2^-$. 

The observed rates of damage to the permeability of membranes and to membrane-bound G3PDH by $\text{H}_2\text{O}_2$ and C2- when simultaneously present (i.e. irradiation in the presence of formate) were only 38% and 33% of the rates predicted from the sum of the effects of these radicals individually (Table 3). These results therefore indicate that interactions between these radicals interfere with the destructive effects of the individual free radicals. In contrast, in the absence of scavengers, irradiation of air-saturated aqueous buffer produces $.\text{OH}$, $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ simultaneously (Tables 1 and 2) and this mixture produced damage at rates greater than the calculated rates, indicating cooperative interactions between the free radicals. Addition of superoxide dismutase to the mixture decreased the rate of
enzymic inactivation to the calculated rate, indicating that no interaction affecting the rate of this damage occurs between H2O2 and .OH (Table 4). However, the rate of increase in permeability is still 50% greater than the calculated value (Table 3). These results suggest that .OH and H2O2 probably interact in an indirect manner towards the membrane and that components of the membrane itself are involved in the interactions which produce the observed enhancement of rate.

3.2. Mixture of oxidizing free radicals (.OH and H2O2) + reducing radicals (e- and H.)

The damage to permeability in anaerobic solutions is quite considerable as shown in Table 1. It is interesting to note that damage in anaerobic solutions is greater than in air-saturated solutions containing formate. This is not surprising in view of the high R(mb) of reducing radicals (Kong 1979). As shown in Table 3, we found no significant difference between the observed and calculated rates of damage to permeability. The rate of inactivation of G3PDH is markedly decreased after the second hour of irradiation (Figure 4) to a constant (72% of initial rate). In
contrast, when pure G3PDH was irradiated, no such effect was observed (Buchanan and Armstrong 1978). The rate of loss of enzymic activity was proportional to dose and reached 60% of its zero-dose activity after 100 krad of irradiation. As pointed out in section 2, anaerobic media contained 25 μM oxygen in the first few minutes. The initial rates of damage may thus be affected by the small amount of O₂-present. This may partly explain the high initial rate of enzymic inactivation in comparison with the calculated rate (Table 4), taking into account the high R(enz) of O₂- and the occurrence of the Haber-Weiss reaction (see discussion). Therefore the rate of inactivation after two hours of irradiation was taken as the anaerobic rate. During this period, all of the oxygen free radicals would have been consumed. Nevertheless, O₂- would not affect the rate of increase in permeability significantly owing to its low R(mb).

3.3. Effects of oxygen on the actions of free radicals

A common feature of the damage in oxygenated solutions is the downward curvature of the graphs of damage to permeability and, to a smaller extent, to membrane-bound
G3PDH. This indicated an acceleration of damage which is absent in air-saturated and anaerobic solutions, except in the case of damage by .OH to membranes in air-saturated solution. Again the kinetics of damage to membrane-bound G3PDH in oxygenated solutions was different than that to permeability, in that curvatures were slight and appeared only in the initial portion of the plots. In fact, the damage in the presence of catalase and dismutase in oxygenated solutions was linear. Rates of damage to permeability and membrane-bound G3PDH in all of the oxygenated solutions were close to those of the corresponding air-saturated solutions (Tables 5 and 6), except in the case of catalase plus dismutase. Therefore the increased concentration of oxygen did not increase the initial rates of damage in most cases. In fact, a seemingly protective effect of oxygen on membranes was observed in the first two hours of irradiation in oxygenated solutions containing no scavengers. In the simultaneous presence of catalase and dismutase, the initial rates are increased by oxygen. The reason for this is not known. However, the high values in the last columns of table 5 and 6 clearly illustrate the immense enhancement of damage due to oxygen in the subsequent period of irradiation. The ratios of
final to initial rates of increase in permeability are an order of magnitude greater than the corresponding ratios for enzymic inactivation, as can be seen by a comparison of the last columns of Tables 5 and 6. This damage to permeability has an overwhelming autocatalytic component while autocatalysis is absent or contributes much less with respect to damage to the enzyme.

In the simultaneous presence of dismutase, catalase, formate and oxygen, virtually all of the radicals formed in radiolysis of water would be removed. Under these circumstances, most of the damage to the ghosts would be due to the direct effect of radiation, together with the relatively small quantities of radicals formed inside the intact ghosts. Organic radicals would be formed in the lipid and proteins of the membranes. The effect of oxygen on the reactivities of these radicals is shown in Figures 1 and 2. The relatively small amount of damage in comparison with other oxygenated solutions reflects a relatively low yield of radicals formed by direct effects of irradiation, rather than the limited reactivity of these radicals.
3.4. Comparison of the protective effects of various free
radical scavengers individually

The relative effectiveness of free radical scavengers in
protecting membranes and G3PDH is listed in descending order
in Tables 1 and 2. As shown by a comparison of Figures 1
and 3 and as reflected in the values of slopes in Table 1,
the scavengers vary in their protective effect to membrane
permeability in descending order of effectiveness as
follows:

formate (90%) > nitrogen (65%) > catalase (60%) > dismutase
(32%)

The percentages in parentheses express the fact that the
scavenger decreases the rate of damage in comparison with
media containing no scavengers, by that percentage. For
instance, the rate in the presence of formate is 10% of that
in the absence of scavengers. Comparison of Figures 2 and
4, and as shown by data in Table 2, the order of
effectiveness with respect to inactivation of G3PDH is:

nitrogen (100%) > formate (77%) > dismutase (48%) = catalase
(44%)
For reasons mentioned in section 3.2, the protective effect of N2 was estimated from the final slope of the curve in Figure 4. Comparison of damage in oxygenated buffers also shows that formate is protective.
4. Discussion

4.1. Interactions among free radicals that enhance damage

The mixture containing H2O2 and .OH was found to produce damage to permeability at a rate twofold greater than that accounted for by the independent actions of the constituent free radicals. Since .OH is highly reactive and reacts at or close to the site at which it is formed, its interaction with H2O2 is not likely. Therefore the enhanced rate would not be due to direct interaction between .OH and H2O2. Powers (1972) has observed similar synergistic effects of H2O2 and .OH in his study of radiation damage to cells. He suggested that organic radicals were produced in the course of abstraction of H by .OH in reaction (5), with .OH then being regenerated by reaction (6).

\[ \text{PH}_2 + .\text{OH} \rightarrow \text{RH} + \text{H}_2\text{O} \]  

\[ \text{RH} + \text{H}_2\text{O}_2 \rightarrow R + .\text{OH} + \text{OH}^- + \text{H}^+ \]  

The current data confirm that these or similar processes take place in cell membranes. Since the organic radicals
formed are probably lipid or protein radicals fixed in the membranes, the reactive species formed in reaction (6) would be confined to the membrane, reacting at the sites of their formation, thus the absence of similar enhancement in damage to G3PDH.

The mixture containing .OH, H2O2, and O2- was also found to produce damage to both permeability and G3PDH at rates greater than the calculated rates. The enhanced rate of increase in permeability (as explicable in terms of the synergistic interactions both between H2O2 and .OH and between H2O2 and O2-. The synergistic effect of .OH and H2O2 alone cannot account for the enhancement in damage observed in this case, since this mixture was observed to produce an enhancement of the rate so great (Table 1) that O2- would have to account for 30% of the enhanced rate if there was no synergistic effect between O2- and .OH. It has been shown that O2- alone could contribute only 13% (Kcnq, 1979). Moreover, the concentration of H2O2 in this mixture (G=0.6) is less than that present in the mixture mentioned in the previous case (G=2.3), and O2- and .OH do not seem to interact synergistically (ratio in Table 3 for catalase is close to 1). Therefore, the Haber-Weiss or some similar
reaction must be invoked to account for part of the enhancement. Since the synergistic effect of OH and H2O2 is absent in inactivation of G3PDH, the enhanced rate of enzymic inactivation is due mainly to the Haber-Weiss reaction, consistent with the smaller enhancement than in the case of permeability (Table 4). Addition of catalase or superoxide dismutase to abolish the Haber-Weiss reaction and thus the accompanying enhancement, yields rates close to the calculated rates for changes both in permeability and enzymic inactivation (Tables 3 and 4). Evidently, in this case the Haber-Weiss reaction plays its conventional destructive role in damage to biological systems. In the next section, in contrast, a situation is described in which the Haber-Weiss reaction paradoxically plays a protective role.

4.2. Haber-Weiss like interactions among free radicals which decrease damage

The mixture of H2O2 and O2- was found to induce damage to both permeability and G3PDH by 60% less than the calculated rate. This observation suggests a moderating reaction between O2- and H2O2. Interactions between H2O2 and O2-
have long been known to occur in biological systems and the Haber-Weiss reaction has been invoked to explain it. If the Haber-Weiss reaction did occur in our case, O₂⁻ and H₂O₂ would be removed by reaction (2) and replaced by .OH, and since formate in the medium removes .OH, the net result would be decrease in the concentrations of O₂⁻ and H₂O₂. Such decreases in the overall concentration of free radicals have a protective effect on the changes in permeability and G3PDH activity in view of the known effectiveness of H₂O₂ in causing damage to the membrane and the known effectiveness of O₂⁻ in inactivating G3PDH (Kong 1979). The present results thus support the occurrence of a Haber-Weiss like reaction in this case, which paradoxically produces a protective effect, since the potent end products of the reaction were removed by formate. Consistent with this explanation, the removal of either O₂⁻ or H₂O₂ from the mixture by superoxide dismutase or catalase restores the effectiveness of the remaining free radical in the medium, as reflected by an increase in damage in the presence of either of these scavengers (Figures 1 and 2, Tables 1 and 2).
It is thermodynamically feasible to produce singlet oxygen by the Haber-Weiss reaction (Koppenol and Butler 1977), and lipid peroxidation due to this reaction has been postulated to be due to singlet oxygen (Kellogg and Fridovich 1975). However, the protective effect of Haber-Weiss reaction in our case suggests that the contribution of singlet oxygen to the damage of membranes is small in comparison with the other free radicals present.

4.3. Interactions between oxidizing and reducing free radicals which moderate damage

Fates of increase in permeability in anaerobic media in the absence of formate are moderated by interactions between oxidizing radicals (·OH + H2O2) and reducing radicals (e− + H·) when both are simultaneously present (Table 1). The calculated rates are close to the observed rates (Table 3). This might mean that the free radicals in the mixture do not interact. However, in view of the enhanced damage to permeability due to the mixture (·OH + H2O2) in aerobic media, we would expect this synergism to be evident anaerobically also. That it is not observed implies, at the least, that the simultaneous presence of reducing free
radicals inhibits the cooperation between H2O2 and .OH. The cessation of damage to membrane-bound G3PDH after 2 hours is related to the presence of membranes or the residual contents of the ghosts, since destruction of the purified enzyme is continuous (Buchanan and Armstrong 1978). Its apparent cessation presumably reflects attainment of a steady state between destruction and reactivation. The residual contents of ghosts would both limit destruction (by virtue of catalase and dismutase) and promote re-reduction of oxidized SH groups (by virtue of reducing enzymes and SH compounds), or by the reducing radicals themselves. We have thus shown that the reducing and oxidizing free radicals may exert an antagonistic effect on each other's actions, thus moderating the damage due to either. This is further supported by the observation that the removal of .OH from this mixture by formate increased inactivation of G3PDH anaerobically.

4.4. Comparison of the sensitivity of enzymic inactivation and permeability to oxygen

It was pointed out in a previous study (Kong 1979) that permeability was more sensitive to oxygen than inactivation
of G3PDH in the absence of scavengers. It was shown that the anaerobic rate of increase in permeability was increased 2.8-fold by the admission of air, but 170-fold by saturation with pure oxygen. The corresponding increases in rates of enzymic inactivation were 1.5- and 4.1-fold. This represents a 60-fold increase in the aerobic rate of change of permeability but only a 2.7-fold increase in the case of enzymic inactivation due to the further increase in concentration of oxygen in the already air-saturated media. Data in the present study further support this difference in sensitivity. In the presence of formate, the anaerobic rate of increase in permeability is increased 2.5-fold and the aerobic rate by 7.3-fold by the admission of pure oxygen, while the corresponding increases in enzymic inactivation are 13- and 4.2-fold respectively. Similarly, 9.5- and 5.5-fold increases in aerobic rates of change in permeability and enzymic inactivation, due to oxygen are observed in the media containing both catalase and dismutase. These comparisons between permeability and enzymic inactivation as concentration of oxygen increases indicate that enzymic inactivation is as sensitive to oxygen as permeability in air-saturated media but becomes relatively insensitive at higher concentrations of oxygen.
In contrast, permeability is more sensitive at higher concentrations of oxygen. This difference in sensitivity is confirmed by the comparison of the last columns of Table 5 with those of Table 6. It can be seen that in relation to permeability the greater the aerobic rate of damage the greater is the enhancement by oxygen, while the converse is true for enzymic inactivation. This lower sensitivity of enzymic inactivation at high concentrations of oxygen seems to suggest that the aerobic rates are close to the maximum possible acceleration of inactivation which oxygen can produce. Thus further increases in the concentration of oxygen enhances the lower aerobic rates significantly but not the already high aerobic rates. Evidently, the differences in sensitivity to oxygen are independent of the nature of free radicals causing the damage, since the use of scavengers to alter the nature of the free radicals present does not significantly alter the above mentioned differential sensitivity. The acceleration by oxygen of the rates of increase in permeability and the absence of such effects in enzymic inactivation in oxygenated buffers (in all 3 cases in Tables 5 and 6) offer further evidence for the mechanisms invoked to explain the enhancement by oxygen of damage to permeability in the second paper in this series.
(Kong 1979), in which the increased branching of chain reactions in the membranes is considered to be the major cause of the acceleration of the rate of damage caused by oxygen. Furthermore the following roles of oxygen in the interactions of free radicals is suggested.

4.5. Mechanism of enhancement by oxygen of damage to membranes

The conversion from an anaerobic to an aerobic (air-saturated) medium involves the replacement of the reducing radicals (e-, H.) by O$_2^-$. This results in a change from a mixture of oxidizing (·OH, H$_2$O$_2$) and reducing radicals to one of predominantly oxidizing radicals (·OH, H$_2$O$_2$, O$_2$-). Such a change in the nature of the free radicals has a profound effect on damage to the system, in three main respects. Firstly, the removal of reducing radicals abolishes their moderating actions on the synergistic effects between H$_2$O$_2$ and ·OH. Secondly, the presence of O$_2$- allows the Haber-Weiss like reactions to take place. Thirdly, O$_2$- has much higher $R(enz)$ than e- (about 15-fold the latter), the replacement will increase enzymic inactivation. The above considerations explain most
of the differences in damage between anaerobic and aerobic samples listed in Tables 5 and 6. In the presence of formate the damage to permeability is less in aerobic than in anaerobic buffer because the replacement of e- by O2- allows the Haber-Weiss reaction to take place, resulting in a decrease in damage since the .OH produced is scavenged by formate. Furthermore, R(mb) of O2- is 0.7-fold that of e- (Kong 1979). Thus the smaller R(mb) may contribute to the decrease in damage but obviously only to a minor extent. However, in the case of damage to G3PDH, since R(enz) of O2- is greater than that of e-, this is at least a partial cause of the greater overall rate of inactivation in aerobic buffer. Damage both to permeability and G3PDH is greater in aerobic buffer than in anaerobic buffer in the absence of scavengers, both because of the occurrence of the Haber-Weiss reaction and the removal of the restraint on the cooperative interaction between H2O2 and .OH.

The effect of any further increase in the concentration of oxygen in going from an air-saturated to an oxygen-saturated medium seems to be due primarily to the interaction of oxygen with the initial sites of primary damage. This is supported by the observation that the rates
of damage in the presence of oxygen are initially similar to those in the presence of air, but increase markedly after the first 2 hours. Moreover, the final rates increase proportionately with the initial amount of damage (column 4, Tables 5 and 6). From the foregoing, we propose a dual mechanism for the "oxygen effect" in irradiated biological systems. Admission of oxygen to an anaerobic system initially causes the conversion of e- and H to C2-. As a result, the cooperative interactions between the oxidizing free radicals such as the Haber-Weiss reaction, are initiated. In addition however, the presence of oxygen accelerates the ensuing degradation of membranes causing greatly increased branching of chain reactions subsequent to initial damage. The mechanisms by which oxygen enhances damage by its interaction with initial damage have been discussed in detail in the second paper of this series (Kong 1979).

4.6. Relative effectiveness of scavengers

The relative effectiveness of the scavengers shown in Tables 1 and 2 allows quantitative prediction of their potential as radioprotective agents IN VIVO. Consistent with the fact
that .OH is the most destructive radical produced during irradiation, formate offers the greatest protection to the the irradiated systems. Catalase and dismutase, though less effective than formate, protect by preventing the Haber-Weiss (or some similar) reaction. The relative effectiveness of catalase and dismutase depends on the relative susceptibility of the system to O2- and H2O2. In our case, catalase protected membranes to a greater extent than dismutase, while dismutase offered greater protection to G3PDH (a SH-enzyme) than catalase. In comparison with formate, nitrogen is not as protective as commonly believed. It is shown in Tables 1 and 2 that N2 is less protective than formate in aerobic media. Also, combinations of scavengers do not always provide more protection than a single scavenger as evidenced by the discussion in section 4.2. Formate does not provide additional protection to permeability under anaerobic conditions. In the case of damage to permeability, formate abolishes the synergistic effects between .OH and H2O2 as well as preventing the damage due to .OH. On the other hand, any decrease in concentration of oxidizing radicals attenuates their moderating effect on reducing radicals which are relatively effective in causing damage to permeability (Kong 1979).
The resultant damage to membranes thus represents a balance between these opposing effects so that formate may prevent or enhance damage. However, in the case of enzymic inactivation, the latter effect is insignificant because $f(enz)$ of reducing radicals is relatively small in comparison with the other free radicals, so that formate is always protective towards G3PDH.
TABLE 1. EFFECTS OF SCAVENGERS ON THE YIELDS OF INDIVIDUAL FREE RADICALS AND ON THE RATES OF INCREASE IN PERMEABILITY.
The yields of free radicals are given in molecules or ions per 100 ev. of radiation. The rates of increase of permeability were calculated from the slopes of plots given in Figures 1 to 6. The rates were expressed as the logarithm of percentage of ghosts remaining intact after each hour of irradiation. Conditions were: 0.01 M. phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mosM., temperature 25 deg C, concentration of ghosts 10% v/v, dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffer solutions saturated by atmospheric air unless oxygen or nitrogen is specified. Catalase, superoxide dismutase or formate when present were at final concentrations of 50 units/ml, 20.4 units/ml, and 10 mm in the buffer solutions. 'Organic radicals' means those formed in the membranes due to direct interaction of membrane components with radiation. The slopes and their standard errors were obtained by regression analysis (see Methods). The standard errors of most of the slopes are less than +/-30%. The standard error of G values are of the order of 4% (Draganic, Nenadovic and Draganic 1969).
<table>
<thead>
<tr>
<th>Scavengers Present</th>
<th>Yields of free radicals</th>
<th>Slope $x 10^{-3}$</th>
<th>$h \mu r^{-1}$</th>
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<tr>
<td></td>
<td>OH H2O2 O2$^-$ e- CO2-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>0.6 6.2</td>
<td>1.70+/-0.53</td>
<td></td>
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<tr>
<td>Formate, dismutase</td>
<td>3.7</td>
<td>3.50+/-1.6</td>
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<td>Formate, catalase</td>
<td>6.2</td>
<td>3.93+/-1.07</td>
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<td>02, catalase</td>
<td>(organic radicals)</td>
<td>4.17+/-1.24</td>
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</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase, dismutase</td>
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<td>5.05+/-1.41 (initial)</td>
<td>15.92+/-5.07 (final)</td>
</tr>
<tr>
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<td>5.45+/-1.33</td>
<td></td>
</tr>
<tr>
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<td>2.8 0.6 2.8 (H) 0.6</td>
<td>5.79+/-0.67</td>
<td></td>
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<tr>
<td>Catalase</td>
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<tr>
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<td>1577+/-8.75 (final)</td>
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TABLE 2. EFFECTS OF SCAVENGERS ON RATES OF INACTIVATION OF MEMBRANE-BOUND G3PDH. The yields of free radicals are given in molecules or ions per 100 ev. of radiation. The rates of damage to activity of G3PDH were calculated from the slopes of plots given in Figures 1 to 6. The rates were expressed in logarithm of percentage of activity remaining after each hour of irradiation. Conditions were as described in Table 1. slopes and their standard errors were obtained by regression analysis (see Methods). The standard errors of most of the slopes are less than +/-20%.
<table>
<thead>
<tr>
<th>Scavengers Present</th>
<th>Yields of Free Radicals</th>
<th>Slope x 10^-3 H</th>
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<td></td>
<td>in G values</td>
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<td>( \cdot \text{OH} )</td>
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<td>25.74 +/- 3.90</td>
<td></td>
</tr>
<tr>
<td>catalase, dismutase</td>
<td>2.8</td>
<td>32.47 +/- 3.68</td>
<td></td>
</tr>
<tr>
<td>O\text{O}_2, catalase, (organic radicals)</td>
<td>35.47 +/- 6.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dismutase, formate</td>
<td>2.8</td>
<td>2.3</td>
<td>43.97 +/- 3.77</td>
</tr>
<tr>
<td>dismutase</td>
<td>2.8</td>
<td>3.4</td>
<td>47.27 +/- 5.96</td>
</tr>
<tr>
<td>catalase</td>
<td>6.2</td>
<td>53.93 +/- 8.76</td>
<td></td>
</tr>
<tr>
<td>N\text{H}_2</td>
<td>2.8</td>
<td>0.6</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>(H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>no scavengers</td>
<td>2.8</td>
<td>0.6</td>
<td>3.4</td>
</tr>
<tr>
<td>O\text{O}_2</td>
<td>2.8</td>
<td>0.6</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O\text{O}_2, formate</td>
<td>0.6</td>
<td>3.4</td>
<td>31.13 +/- 8.86 (initial)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O\text{O}_2, catalase, dismutase</td>
<td>2.8</td>
<td>178.93 +/- 25.83</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 3. SUMMARY OF EFFECTS OF INTERACTIONS BETWEEN FREE RADICALS ON THE RATES OF INCREASE OF PERMEABILITY. Rates from Table 1 are listed in columns 2. Values in column 3 are calculated by summing the products of \( R(mb) \) and \( G \) of each kind of free radicals in the mixture. \( R \) represents the rate of damage per molecule or ion of free radical and \( G \) is the radical yield in molecules or ion per 100 ev. of energy. Numbers in column 4 are obtained by dividing numbers of column 2 by those of column 3. Conditions were as described in Table 1. The rates in columns 2 and 3 were compared by Student's t test for equality. The test showed that the ratios with (*) (i.e. buffers containing formate, or dismutase, or no scavengers) are different from unity at 95% confidence level. The remaining two ratios are not significantly different from unity within the same confidence limits. The relatively large standard errors for the ratio result inevitably from the summation of the relative errors of the two values from which the ratio was derived.
<table>
<thead>
<tr>
<th>scavengers present</th>
<th>rate of increase in permeability during irradiation x 10^-3 hour^-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>formate</strong></td>
<td>experimental: 1.70±0.53, calculated: 4.48±1.67, exp/calc: 0.38±0.26</td>
</tr>
<tr>
<td><strong>N2</strong></td>
<td>experimental: 5.79±0.67, calculated: 8.60±3.07, exp/calc: 0.67±0.32</td>
</tr>
<tr>
<td><strong>catalase</strong></td>
<td>experimental: 6.63±0.72, calculated: 7.18±2.56, exp/calc: 0.92±0.43</td>
</tr>
<tr>
<td><strong>dismutase</strong></td>
<td>experimental: 11.26±1.76, calculated: 7.23±2.97, exp/calc: 1.56±0.89</td>
</tr>
<tr>
<td>no scavengers</td>
<td>experimental: 16.45±3.15, calculated: 7.75±2.86, exp/calc: 2.12±1.19</td>
</tr>
</tbody>
</table>
TABLE 4. EFFECTS OF INTERACTIONS BETWEEN FREE RADICALS ON THE RATES OF DAMAGE TO MEMBRANE-BOUND G3PDH. Rates from Table 2 are listed in column 2. Numbers in column 3 are calculated by summing the products of R(mb) and G of each kind of free radicals in the mixture. R represents the rate of damage per molecule or ion of free radical and G is the radical yield in molecules or ion per 100 ev. of energy. Values in column 4 are obtained by dividing values in column 2 by those of column 3. Conditions were as described in Table 1. The rates were compared as in Table 3. At the 95% confidence level, the ratios with (*) are significantly different from 1 while the remaining two ratios are not significantly different from unity.
TABLE 4. SUMMARY OF THE EFFECTS OF INTERACTIONS BETWEEN FREE RADICALS ON THE RATES OF INACTIVATION OF MEMBRANE-BOUND G3PDH.

<table>
<thead>
<tr>
<th>Scavengers present</th>
<th>Rate of inactivation of G3PDH during irradiation x 10^{-3} hour⁻¹</th>
<th>Experimental</th>
<th>Calculated</th>
<th>Exp/Calc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate</td>
<td>19.24+/−4.12</td>
<td>58.70+/−13.94</td>
<td>0.33+/−0.16</td>
<td></td>
</tr>
<tr>
<td>N₂</td>
<td>0.0</td>
<td>39.68+/−8.23</td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Catalase</td>
<td>47.27+/−5.36</td>
<td>62.03+/−13.26</td>
<td>0.76+/−0.25</td>
<td></td>
</tr>
<tr>
<td>Dismutase</td>
<td>43.97+/−3.77</td>
<td>50.97+/−10.05</td>
<td>0.86+/−0.25</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>84.28+/−7.99</td>
<td>66.85+/−14.27</td>
<td>1.25+/−0.38</td>
<td></td>
</tr>
<tr>
<td>Scavengers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 5. THE EFFECTS OF OXYGEN ON THE RATES OF INCREASE IN PERMEABILITY OF GHOSTS. For nonlinear plots the initial and final rates are given with the final rates immediately below the initial rates. Unfortunately, no data were obtained for catalase in anaerobic buffer for both permeability and enzymic inactivation. The initial rates in air-saturated and oxygenated buffers were analysed by ANCOVA1 to test for equality. At the 95% confidence level, it was found that the ratios with (*) are significantly different from 1.
TABLE 5. THE EFFECTS OF OXYGEN ON THE RATES OF INCREASE IN PERMEABILITY OF GHOSTS.

<table>
<thead>
<tr>
<th>scavengers</th>
<th>rates of increase of permeability $\times 10^{-3}$</th>
<th>comparison of rates during under the specified irradiation atmospheres in irradiation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>present</td>
<td>during</td>
</tr>
<tr>
<td>N2</td>
<td>air</td>
<td>O2</td>
</tr>
<tr>
<td>formate</td>
<td>5.45</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>(initial)</td>
<td>(initial)</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
<td>2.5+/-1.6</td>
</tr>
<tr>
<td></td>
<td>(final)</td>
<td>(final)</td>
</tr>
<tr>
<td>nC</td>
<td>5.79</td>
<td>16.45</td>
</tr>
<tr>
<td></td>
<td>(initial)</td>
<td>(initial)</td>
</tr>
<tr>
<td>scavengers</td>
<td>986</td>
<td>170+/-60</td>
</tr>
<tr>
<td></td>
<td>(final)</td>
<td>(final)</td>
</tr>
<tr>
<td>catalase,</td>
<td>5.0</td>
<td>47.50</td>
</tr>
<tr>
<td></td>
<td>(initial)</td>
<td>(initial)</td>
</tr>
<tr>
<td>dismutase</td>
<td>15.92</td>
<td>1577</td>
</tr>
<tr>
<td></td>
<td>(final)</td>
<td>(final)</td>
</tr>
</tbody>
</table>
TABLE 6. THE ENHANCEMENT BY OXYGEN ON THE ANAEROBIC AND AEROBIC RATES OF INACTIVATION OF MEMBRANE-BOUND G3PDH.

Analysis of data is discussed in legend of Table 5. At 95% confidence level, it was found that the ratios of initial rates in air-saturated and oxygenated buffers were similar to + in all cases except the ratio with (*).
<table>
<thead>
<tr>
<th>scavengers</th>
<th>rates of inactivation of G3PDH x 10^{-3}</th>
<th>comparison of rates under the specified irradiation atmospheres in irradiation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>air</td>
<td>O2</td>
</tr>
<tr>
<td>formate</td>
<td>6.40</td>
<td>19.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>55.5</td>
<td>84.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scavengers</td>
<td>229.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>catalase</td>
<td>32.47</td>
<td>178.9</td>
</tr>
<tr>
<td>dismutase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 1. EFFECTS OF MIXTURES OF .OH, H2O2 AND C2- ON THE PERMEABILITY OF RESEALED ERYTHROCYTE GHOSTS. Logarithm of percentage of ghosts remaining intact after each hour of irradiation is plotted as a function of time in hour. Conditions were: 0.01 M. phosphate buffer pH 7.4 rendered isotonic by the addition of NaCl to 300 mosM., temperature 25 deg C, concentration of ghosts 10% v/v, dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffer solutions saturated by atmospheric air unless oxygen or nitrogen is specified. Catalase, superoxide dismutase or formate when present were at final concentrations of 50 units/ml, 20.4 units/ml, and 10mM in the buffer solutions. The slopes and their standard errors were obtained by regression analysis as described in the Methods.

Symbols

- .OH + H2O2 + O2-
- H2O2 + O2-
- .OH + H2O2
- .OH + O2-
- Organic radicals + O2
FIGURE 1. EFFECTS OF MIXTURES OF OH, H2O2 AND C2- ON THE PERMEABILITY OF RESEALED ERYTHROCYTE GHOSTS.
FIGURE 2. EFFECTS OF MIXTURES OF .OH, H2O2 AND C2- ON THE ACTIVITY OF MEMBRANE-BOUND G3PDH OF RESEALED ERYTHROCYTE GHOSTS. Logarithm of percentage of activity of G3PDH remaining after each hour of irradiation is plotted as a function of time in hour. Conditions were as described in Figure 1.

Symbols

.\*OH + H2O2 + C2- \(\bigcirc\)
H2O2 + O2- \(\square\)
.\*OH + H2O2 \(\times\)
.\*OH + O2- \(\diamond\)
Organic radicals + O2 \(\ast\)
FIGURE 2. EFFECTS OF MIXTURES OF OH, H2O2 AND C2- ON THE ACTIVITY OF MEMBRANE-BOUND G3PDH OF RESEALED ERYTHROCYTE GHOSTS.
FIGURE 3. EFFECTS OF MIXTURES OF OXIDIZING FREE RADICALS: \( \cdot \text{OH} \) AND \( \text{H}_2\text{O}_2 \) AND REDUCING FREE RADICALS: \( e^- \), \( \cdot \text{H} \) AND \( \text{CC}^2- \) ON THE PERMEABILITY OF RESEALED ERYTHROCYTE GHOSTS. Logarithm of percentage of ghosts remaining intact after each hour of irradiation is plotted as a function of time in hour. Conditions were as described in Figure 1.

Symbols

\[
\begin{align*}
\cdot \text{OH} + \text{H}_2\text{O}_2 + e^- + \cdot \text{H} & \quad \times \\
\text{H}_2\text{O}_2 + e^- + \text{CC}^2- & \quad \ast
\end{align*}
\]
FIGURE 3. EFFECTS OF MIXTURES OF OXIDIZING FREE radicals: \( \cdot \mathrm{CH} \) AND \( \mathrm{H}_2\mathrm{O}_2 \) AND REDUCING FREE radicals: \( \cdot \mathrm{e}^- \), \( \cdot \mathrm{H} \) AND \( \mathrm{CO}_2^- \) ON THE PERMEABILITY OF RESEASED ERYTHROCYTE GHOSTS.
FIGURE 4. EFFECTS OF MIXTURES OF OXIDIZING FREE RADICALS: \( \cdot \text{OH} \) AND \( \text{H}_2\text{O}_2 \) AND REDUCING FREE RADICALS: \( \text{e}^- \), \( \cdot \text{H} \) AND \( \text{CO}_2^- \) ON THE ACTIVITY OF MEMBRANE-BOUND G3PDH OF RESEALED ERYTHROCYTE GHOSTS. Logarithm of percentage of activity of G3PDH remaining after each hour of irradiation is plotted as a function of time in hour. Conditions were as described in Figure 1.

Symbols

\[
\begin{align*}
\cdot \text{OH} + \text{H}_2\text{O}_2 + \text{e}^- + \cdot \text{H} & \quad \times \\
\text{H}_2\text{O}_2 + \text{e}^- + \text{CO}_2^- & \quad \star
\end{align*}
\]
FIGURE 4. EFFECTS OF MIXTURES OF OXIDIZING FREE RADICALS: \( \cdot \text{OH} \) AND \( \text{H}_2\text{O}_2 \) AND REDUCING FREE RADICALS: \( \cdot \text{e}^- \), \( \cdot \text{H} \) AND \( \text{CO}_2^- \) ON THE ACTIVITY OF MEMBRANE-BOUND G3PDH OF RESEALED ERYTHROCYTE GHOSTS.
FIGURE 5. EFFECTS OF OXYGEN ON THE DAMAGE BY .OH, H2C2, O2-
AND MIXTURES OF THESE RADICALS ON THE PERMEABILITY OF
RESEALED ERYTHROCYTE GHOSTS. Logarithm of percentage of
ghosts remaining intact after each hour of irradiation is
plotted as a function of time in hour. Conditions were as
described in Figure 1.

Symbols

. OH + H2O2 + C2-  
. OH + H2O2 + C2- + O2  
H2O2 + O2-  
H2O2 + O2- + O2  
. OH  
. OH + O2  

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FIGURE 5. EFFECTS OF OXYGEN ON THE DAMAGE BY \( \cdot \text{OH} \), \( \text{H}_2\text{O}_2 \), \( \text{O}_2 \)-
AND MIXTURES OF THESE RADICALS ON THE PERMEABILITY OF
RESEALED ERYTHROCYTE GHOSTS.

![Graph showing effects of oxygen on the damage by \( \cdot \text{OH} \), \( \text{H}_2\text{O}_2 \), \( \text{O}_2 \)-
and mixtures of these radicals on the permeability of
resealed erythrocyte ghosts.](image-url)
FIGURE 6. EFFECTS OF OXYGEN ON THE DAMAGE BY ·OH, H2O2, O2-
AND MIXTURES OF THESE RADICALS ON THE ACTIVITY OF
MEMBRANE-BOUND G3PDH. Logarithm of percentage of activity
of membrane-bound G3PDH remaining after each hour of
irradiation is plotted as a function of time in hour.
Conditions were as described in Figure 1.

Symbols

·OH + H2O2 + O2-
·OH + H2O2 + C2- + O2
H2O2 + O2-
H2O2 + O2- + O2
·OH
·OH + O2
FIGURE 6. EFFECTS OF OXYGEN ON THE DAMAGE BY .OH, H2O2, O2-
AND MIXTURES OF THESE RADICALS ON THE ACTIVITY OF
MEMBRANE-BOUND G3PDH.
BIBLIOGRAPHY


Conclusion

This thesis was divided into four major sections. In section 1, it was shown that oxygen may either enhance or decrease the damage caused by free radicals, depending on the nature of the system. In section 2, we found that oxygen invariably potentiated free-radical damage to isolated cell membranes and a membrane-bound SH enzyme, G3PDH. In section 3, .OH was confirmed to be the most destructive species to both membranes and enzyme. Nevertheless, the other radicals, though different in redox potentials and reactivities, were not significantly different from each other in causing damage to the membranes. Therefore, a wide variety of reactions contributes to the breakdown of the membrane's permeability barrier. Damage to G3PDH was almost exclusively caused by the oxidizing radicals, .OH, O2- and H2O2 and the extent of damage varied according to the reduction potentials of these radicals.

In section 4, interactions were found to occur among primary free radicals, probably mediated by secondary radicals formed in the membranes. Three major types of interactions
were observed: (i) Haber-Weiss like reactions between O2- and H2O2, (ii) synergistic interactions between H2O2 and .OH, and (iii) antagonistic interactions between the oxidizing and reducing radicals. Hence free radical scavengers may be protective or destructive depending on their effect on these interactions. Formate was most protective because it removed the most destructive .OH, while catalase and dismutase protected the membranes by prohibiting the Haber-Weiss like reactions.

From these combined results, it can be concluded that when oxygen enhances damage, it does so by modifying the initial damage afflicted by free radicals, independent of the nature of the radicals and also by converting reducing radicals to O2-, thus allowing Haber-Weiss like reactions to take place.
APPENDICES

Appendix 1

PREPARATION OF RESEASED HUMAN ERYTHROCYTE GHOSTS

1.1. Prewashing

150 ml of blood was centrifuged in six 40ml centrifuge tubes. The serum and buffy coat were aspirated off and 45 ml of spinned blood was then transferred to a 250 ml centrifuge bottle containing 200 ml of isotonic buffer (5mM sodium phosphate made isotonic with 155mM NaCl, pH 7.4). It was then centrifuged for 10 min. at 12,000 rpm. The supernatant was decanted. The red blood cells were washed twice in this way.

1.2. Hemolysis of red blood cells

Washed blood was added to 1.4 litres of hypotonic buffer at 4 deg C (10mM sodium phosphate, 0.01mM CaCl2 and 4mM MgCl2, pH 7.4) in a 2 litres beaker, stirred gently for 10 min. and centrifuged at 12,000 rpm for 15 min.
1.3. Resealing

The membranes obtained from hemolysed blood cells were rinsed into a 2 litres beaker with 1.4 litres of 37 deg C isotonic buffer and incubated for 60 min. The resulting resealed ghosts were then centrifuged at 16,000 rpm for 10-15 min. in 250 ml bottles. The spun down membranes were collected in a 40 ml centrifuge tube and centrifuged in isotonic buffer. This served as the last wash. This would normally yield a maximum of 16 ml packed membranes.

4 ml of packed membranes were pipetted into 36 ml isotonic buffer in a 40 ml glass tube. This would make 40 ml of 10% v/v membrane suspension for irradiation.
ASSAY OF SUPEROXIDE DISMUTASE

Reagents:

0.05 M potassium phosphate buffer, pH 7.8 containing 0.1 mM EDTA
0.3 mM ferricytochrome C (3.9 mg/ml buffer)
1.5 mM xanthine

Xanthine oxidase in buffer sufficient to cause a rate of reduction of cytochrome C at 550 nm of 0.025 A/min.

Method

The method of assay depends on the ability of superoxide dismutase to compete with ferricytochrome C for O2-generated by aerobic xanthine oxidase system, thus inhibiting the rate of reduction of cytochrome C by this system.
Procedure

Into a 10 mm cell place the following at 25 deg C:

2.7 ml buffer
0.1 ml cytochrome C
0.1 ml xanthine
0.1 ml xanthine oxidase

Record the increase in absorbance at 550 nm for 3 min at 25 deg C. Repeat with different dilutions of xanthine oxidase until a rate of 0.025 A/min is obtained. Then repeat the assay using 0.1 ml of this dilution of xanthine oxidase, 2.6 ml buffer and 0.1 ml of different dilutions of superoxide dismutase until a rate of 0.0125 A/min is obtained. Start the reaction by adding xanthine oxidase last.

The activity of superoxide dismutase (SOD) is calculated by:

\[
\text{units/mg} = \frac{\text{dilution of SOD causing a rate of } 0.0125/\text{min}}{0.1 \times \text{mg SOD/ ml original solution}}
\]
ASSAY OF CATALASE

Reagent

0.05 M potassium phosphate buffer, pH 7.0
H2O2, dilute 0.1 ml H2O2 (30%) to 50 ml using above buffer
so that absorbance at 240 nm is 0.5-0.55.
catalase, 5-10 ug/ml in buffer.

Procedure

Into 10 mm quartz cell place the following at 25 deg C:

2.9 ml H2O2
0.1 ml catalase

Record the rate of decrease of absorbance at 240 nm against
buffer and use the linear portion of the reaction for the
rate.
Activity is calculated as follows,

\[
\text{rate of decrease of absorbance at 240 nm} = \frac{\text{unit/mg}}{\text{mg enz/ml reaction mixture}} \times 0.043 \times 10^3
\]

The molar absorption coefficient for H2O2 is 0.043 x 10^3
Appendix 4

EFFECTS OF TRITON X-100 AND DITHIOCREITOL ON THE ACTIVITY OF MEMBRANE-BOUND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE.

1. Introduction.

Triton X-100 has been routinely used to solubilise membranes to ensure complete accessibility of G3PDH in the assessment of total enzymic activity of the ghost suspension in our experiments. It has been known that it affects the activity of G3PDH. Therefore it is necessary for us to determine the optimal concentration of Triton X-100 that would cause the least inhibition on the enzyme and yet sufficient to solubilise all of the ghosts in the suspension.

It is commonly observed in assay of tissue G3PDH that the activity of the enzyme is often lost due to oxidation of its enzymic SH groups (Tice and Haugaard 1972). Cysteine-HCl is commonly used to restore the activity (Cori, Slein and Cori 1947, Steck and Kant 1974). Dithiothreitol recently prepared by Claland (1963) proved to be a better protective
agent for SH groups because of its low redox potential
(-0.33 v. at pH 7). It is capable of maintaining
monothiols completely in the reduced state and of reducing
disulphides quantitatively. Besides, it is highly soluble
in water and has little tendency to be oxidised by air.
Therefore we prefer to use DTT in our G3PDH assay. In the
present study we will also establish the optimal
concentration of DTT to produce the maximum restoration of
enzymic activity.
2. Experimental

2.1 Materials

Glyceraldehyde-3-phosphate (G3P), dithiothreitol (DTT) and beta-NAD were supplied by Sigma Chemicals Ltd. Sodium arsenate, sodium pyrophosphate, sodium formate and Triton X-100 were supplied by Fisher Chemicals Ltd.

2.2. Methods

2.2.1. Assay

0.1 ml of ghost suspension was added to a cuvet containing the assay reagent except G3P. 0.1 ml of G3P was added and the solution was mixed rapidly. The reaction was followed spectrophotometrically at 340 nm. at 25 deg C.

Taking the addition of G3P as the starting point of the reaction, the increase in absorbance between the first and second minute of reaction was taken as a measure of enzymic activity. This increment is not linear with time but is linear with respect to enzyme concentration in the range indicated. Pyrophosphate is known to inactivate the enzyme which can be reactivated by DTT.
2.2.2. Assay reagents

1.0 ml isotonic buffer pH 7.4
1.4 ml sodium pyrophosphate 30 millimolar, adjusted to pH 8.4 with HCl.

0.1 ml sodium arsenate 0.4 molar
0.1 ml beta-NAD 20 millimolar
0.2 ml Triton X-100 0.25%v/v
0.1 ml DTT 12 millimolar
0.1 ml membrane suspension 10%v/v
0.1 ml G3P 20 millimolar, prepared from barium diethylacetal salt of G3P according to the method by Sigma Chemicals Ltd.

2.2.3. Determination of optimal concentrations of Triton X-100 and DTT

Different concentrations of Triton X-100 were made up in isotonic buffer in terms of volume%. Excess DTT was used in the assay to ensure maximum restoration of enzymic activity. DTT at 12 millimolar was used. After the optimal
concentration of Triton X-100 was determined, this concentration of Triton X-100 would be used in assay of G3PDH activity at different concentrations of DTT which was made up in distilled water.

3. Results.

3.1. Effects of Triton X-100

The rate of reduction of NAD catalysed by G3PDH is not linear with time because of inhibition by products of the reaction, NADH and glyceroyl phosphate (Furfine and Velick 1965). The initial rate of the reaction is therefore measured by the increment of absorbance at 340 nm. between the first and the second minute. Within this range the rate is proportional to the concentration of enzyme. The effects of Triton X-100 on the initial rate and on the rate of inhibition by products of G3PDH were shown in Figures 1 and 2 respectively. The decrease in initial rates of reaction at concentrations of Triton X-100 greater than 0.2% indicated inhibition of the enzymic activity by Triton X-100. At these high concentrations, all the molecules of G3PDH in the ghost suspension were accessible to externally
added substrates because the membranes would be completely solubilised. The solubilisation of membranes could be confirmed visually as the appearance of the ghost suspension turned from turbid to clear when the membranes were dissolved. The rate of inhibition by the products could be estimated from the slope of the plot in Figure 1 at range of 0.2% to 5%. The rate was 0.05 per minute per unit percent increase in concentration of Triton X-100. The rapid increase in initial rates when concentration of Triton X-100 was increased towards 0.2% was mainly due to the increase in accessible enzyme units as greater amount of membranes was dissolved. In this range of concentrations, the suspension remained turbid indicating some undissolved membranes. The shape of the plot in Figure 1 was then due to the effect of two opposing factors: (1) decrease in initial rates due to inhibition by Triton X-100, and (2) increase in initial rates due to increase in accessible enzyme units. At detergent concentrations greater than 0.2% Triton X-100, only the first factor would be present.

Figure 2 showed that Triton X-100 did not affect the rate of product inhibition on the enzyme since the slopes of all the plots were similar.
3.2. Effects of DTT

Enzymic activity was rapidly restored when concentration of DTT was increased from 50 to 60 micromolar (Figure 3). Maximum restoration of activity was reached about 80 micromolar. No significant increase was observed up to 1 millimolar. Therefore any concentration of DTT greater than 80 micromolar would be sufficient to restore most of the activity of the enzyme. Besides restoring the enzymic activity, DTT also suppressed the rates of product inhibition. This was shown by the change in gradients of the plots due to different concentrations of DTT in Figure 4. Comparison of gradients in table 1 indicated that concentrations of DTT from 60 to 100 uM suppressed the rate of product inhibition by 88%. By increasing the initial rate and suppressing product inhibition, DTT increased the sensitivity of the assay due to the increase in the increment of absorbance between the first and second minute.
4. Conclusion.

Triton was found to decrease the initial rate of reaction but did not affect the product inhibition of G3PDH. The optimal concentration of Triton X-100 recommended for the assay was 0.25%. DTI not only restored the enzymic activity but also suppressed product inhibition, thereby increasing the sensitivity of the assay.
FIGURE 1. EFFECT OF TRITON ON THE INITIAL RATES OF REDUCTION OF NAD CATALYSED BY MEMBRANE-BOUND G3PDH.

Absorbance per minute was plotted as a function of concentrations of Triton X-100 in % v/v. The conditions were as described in Methods.
FIGURE 1. EFFECT OF TRITON ON THE INITIAL RATES OF REDUCTION OF NAD CATALYSED BY MEMBRANE-BOUND GAPDH.
FIGURE 2. EFFECT OF TRITON ON THE RATE OF PRODUCT INHIBITION ON MEMERANE-BOUND G3PDH. \((A_{\text{max}}-A)/t\) was plotted against \((A_{\text{max}}-A)\) for various concentrations of Triton X-100. Amax is the maximum absorbance at which NAD in the reaction mixture was exhausted. A is the absorbance at time t.

Conditions were as described in Methods. Concentrations of Triton X-100 were 0.08%, 0.1%, 0.2%, 0.3% 0.5%, 1%, 2%, 5%, 10%.

Symbols

- 0.08%
- 0.1%
- 0.2%
- 0.3%
- 0.5%
- 1.0%
- 2.0%
- 5.0%
- 10%
FIGURE 2. EFFECT OF TRITON ON THE RATE OF PRODUCT INHIBITION ON MEMBRANE-BOUND G3PDH.
FIGURE 3. EFFECT OF DTT ON INITIAL RATE OF REDUCTION OF NAD CATALYSED BY MEMBRANE-BOUND G3PDH. Absorbance per minute was plotted as a function of concentrations of DTT in micromolar. Conditions were as described in Methods.
FIGURE 3. EFFECT OF DTT ON INITIAL RATE OF REDUCTION OF NAD
CATALYSED BY MEMBRANE-BOUND G3PDH.
FIGURE 4. EFFECT OF DTT ON THE RATE OF PRODUCT INHIBITION ON MEMBRANE-BOUND G3PDH. \((A_{\text{max}} - A)/t\) was plotted against \((A_{\text{max}} - A)\) for various concentrations of DTT. \(A_{\text{max}}\) and \(A\) were as defined in Figure 2. Conditions were as described in Methods. Concentrations of DTT were 20, 30, 40, 50, 60, 80 and 100 micromolar.

Symbols

- 20 micromolar: +
- 30 micromolar: •
- 40 micromolar: ■
- 50 micromolar: ■
- 60 micromolar: □
- 80 micromolar: △
- 100 micromolar: ★
FIGURE 4. EFFECT OF DTT ON THE RATE OF PRODUCT INHIBITION ON MEMBRANE-BOUND G3PDH.
TABLE 1. EFFECT OF DTT ON THE RATE OF PRODUCT INHIBITION ON MEMBRANE-BOUND G3PDH. \((A_{\text{max}} - A)/t\) was plotted against \((A_{\text{max}} - A)\) for various concentrations of DTT. \(A_{\text{max}}\) is the maximum absorbance at which NAD in the reaction mixture was exhausted. \(A\) is the absorbance at time \(t\). Conditions were as described in Methods. The slopes and their standard errors were obtained by regression analysis using the APL statistical package 'STP2'.
**TABLE 1. EFFECT OF DTT ON THE RATE OF PRODUCT INHIBITION ON MEMBRANE-BOUND G3PDH.**

<table>
<thead>
<tr>
<th>concentrations of DTT (uM)</th>
<th>slopes (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>60.4 +/- 4.6</td>
</tr>
<tr>
<td>30</td>
<td>73.8 +/- 13</td>
</tr>
<tr>
<td>40</td>
<td>69.3 +/- 4.5</td>
</tr>
<tr>
<td>50</td>
<td>23.2 +/- 4.7</td>
</tr>
<tr>
<td>60</td>
<td>6.8 +/- 1.1</td>
</tr>
<tr>
<td>80</td>
<td>10.1 +/- 0.3</td>
</tr>
<tr>
<td>100</td>
<td>7.4 +/- 0.2</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


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