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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

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B.Sc., Hong Kong University, 1973

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

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MASTER OF SCIENCE

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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 resealed erythrocyte ghosts

Author:

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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 $H2O2$, $.OH$, $O2-$
and $e-$; (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 inactivaction of membrane-bound G3P dehydrogenase
(G3PDH). The radiolysis of water by gamma-irradiation was
used as the source of free radicals: $H2O2$, $.OH$, $O2-$ and $e-$.

Ghosts were more susceptible to irradiation than
erythrocytes by an order of magnitude.

Using appropiate mixtures of scavengers such as catalase,
superoxide dismutase and formate, the rates of increase in
permeability (R(mb)) and loss of activity of G3PDH (R(enz))
per molecule of each kind of free radicals were measured.
R(enz) of O2- and H2O2 were 12-fold, and of .OH 17-fold that
of e-. Hence with respect to R(enz): .OH > O2- = H2O2 >
e-. R(mb) of e- and H2O2 were about 1.5-fold and of .OH
3-fold that of O2-. Hence, with respect to R(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 uM to 1150 uM 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 uM) the
latter is more sensitive at higher concentrations of oxygen
(above 230 uM). 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 (Fridovich 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_2O_2 are formed as electrons are added one by one as shown in the following scheme:

$$O_2 + e^- \longrightarrow O_2^-$$

$$O_2^- + e^- + 2H^+ \longrightarrow H_2O_2$$

$$H_2O_2 + e^- \longrightarrow .OH + OH^-$$

$$.OH + e^- + H^+ \longrightarrow H_2O$$
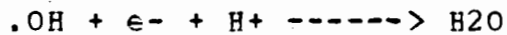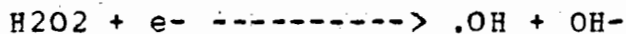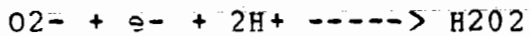
These intermediates are free radicals, which are chemical species having a lone unpaired electron in an outer orbital. The strong tendency to pair this lone electron makes free radicals very reactive. Undoubtedly, the accumulation of

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 sulphydryl 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, erythropoietic
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 .OH, O2- and
H2O2 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 .OH, H2O2, .H and 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 pcduced simultaneously. Therefore, effects of individual radicals and various mixtures of free radicals can be studied. Gamma-irradiation in ccmbination with free radical scavengers was thus chosen in cur 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 tc papers in preparation for or submitted for publication.

In section 1 we show an ambivalent role cf oxygen in either enhancing cr protecting against free radical damage, and show membranes as one cf 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 cn 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.

BIBLIOGRAPHY

Fridovich, I., 1975, American Scientist, 63:54-59. Oxygen: boon and bane.

Singh, A., 1978, Photochem.Photobiol., 28:429-433. Introduction: interconversion of singlet oxygen and related species. The International conference on singlet oxygen and related species in chemistry and biology.

SECTION 1

FUNCTIONS OF OXYGEN IN FREE RADICAL DAMAGE TO BIOLOGICAL SYSTEMS: EVIDENCE FOR A DUAL ROLE AS BOTH SENSITIZER AND PROTECTOR

by

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ABSTRACT

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 analagous 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 anaemias (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 methaemoglobinaemia (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 methaemologlobin 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 cf 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 krads 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 analagous manner:

$$O2- + .OH ----> O2 + OH-$$
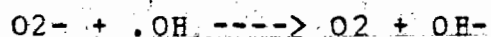
This reaction has been proposed to account for a supression
of the yield of superoxide under conditions in which
formation of hydroxyl radicals is increased (37) during the
irradiation of oxygenated water. The results presented here
suggest the converse: i.e. under the conditions of our
experiments, the presence of superoxide radicals suppressed
the production and action of hyroxyl 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 photolysed water, and they too 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 propogation 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

1. Haugaard, N.   (1968) Physiological Reviews.   48,
    311-375. Cellular mechanisms of oxygen toxicity.

2. Gilbert, D.   (1972) Anesthesiol, 37, 100-111. Oxygen
    and life.

3. Demopoulos, H.B.   (1973) Fed.   Proc.   32,1859-1861. The
    basis of free radical pathology.

4. Pryor, W. (1973) Fed.   Proc.   32, 1862-1869.
    Free-radical reactions and their importance in
    biochemical systems.

5. Slater, T.F.   (1972) Free radical mechanisms in tissue
    injury.(Pion Press, London).

6. Myers, L.S. (1973) Fed. Proc. 32, 1882-1894. Free
    radical damage to nucleic acids and their components by
    ionising radiation.

7. Milvy, P.   (1973) Fed.   Proc.   32, 1895-1902. Control
    of free radical mechanisms in nucleic acid systems:
    studies in radioprotection and radiosensitization.

8. Shohet, S., (1972) New Eng. J. Med. 286, 577-644.
    Hemolysis and changes in erythrocyte membrane lipids.

9. Valentine .W.   (1972) Ann.   Rev. Med. 23, 93-100
    Red-cell enzyme deficiencies as a cause of haemolytic
    disorders.

10. Orr. C (1971) Biochemistry 6, 2995-3000. Studies on
    ascorbic acid. I. factors influencing the
    ascorbate-mediated inhibition of catalase.

11. Davison, A.J. and Wainio, W.W.   (1965) J. Biol.
    Chem. 243, 5023-5027. Reactions of oxygenated
    cytochrome oxidase.

12. Hendriksen, T.   (1967) Radiat.   Res.   32 892-904.
    Effect of oxygen on radiation-induced free radicals in
    proteins.

13. Shanklin, D.R.   (1969) Perspect. Biol. Med.   13,
    80-100. Oxygen toxicity in man.

14. McCord, J.M., Keele, B.B. Jr., and Fridovich, I.
    Proc. Nat. Acad. Sci. U.S. 68 (1971) 1024-1027. An
    enzyme-based theory of obligate anaerobiosis: the
    physiological function of superoxide dismutase.

15. Gregory, E. and Fridovich, I.   (1973) J. Bacteriol.
    114, 1193-1197. Oxygen toxicity and superoxide
    dismutase.

16. Gray, L.H. and Scott, O.C.A. In: Dickens, F. Oxygen
    in the animal organism. (1964), 537-553. Oxygen
    tension and the radiosensitivity of tumours.

17. Draganic, I. and Dragonic, Z. The radiation Chemistry
    of water. Academic Press, New York (9171).

18. Bacq, Z.M. and Alexander, P. In: Dickens, F., Oxygen
    in the Animal Organism. (1964) 509-535.

19. Alexander P.   (1957) Radiat. Res. 6, 653-660. Effect
    of oxygen on inactivation of trypsin by the direct
    action of electrons and alpha particles.

20. Palcic, B. and Skarsgard, L.D.   (1972) Int. J.
    Radiat. Biol. 21, 417-433. The effect of oxygen on
    DNA single strand breaks produced by ionising radiation
    in mammalian cells.

21. Epp, E.R., Weiss, H., Djordjevic, B., and Santomasso,
    A. (1972). Radiat. Res. 52, 321-332. The
    radiosensitivity of cultured mammalian cells exposed to
    single high intensity pulses of electrons in various
    concentrations of oxygen.

22. Asbell, S.O. and Kramer, S. (1971) Radiology 98, 678-681. Oxygen effect on production of radiation-induced myelitis in rats.

23. Coy, P. and Dolman, C.I. (1971) Brit. J. Radiology 44, 705-707. Radiation myelopathy in relation to oxygen level.

24. Raymond, S. and Weintraub, L. (1952) Science 130, 711. Acrylamide gel as a supporting medium for zone electrophoresis.

25. Folkman, J., Conn, H., and Harmel, R. (1972) Science 180, 170-172. Acrylamide polymerisation: new method for determining oxygen content in blood.

26. Folkman, J. (1972). J. Exptl. Med. 133, 275 Measurement of oxygen content in blood and other liquids by oxygen inhibition of chemical reactions.

27. Goldstein, G.M., and Doull, J. (1972) Proc. Soc. Exptl. Biol. Med. 138, 137-139. Treatment of nitrite-induced methemoglobinemia with hyperbaric oxygen.

28. Augenstine, L.G. (1962) Adv. Enzymol. 24, 359-413. The effect of ionising radiation on enzymes.

29. Butler, J., and Robins, A. (1960) Nature 186, 697-698. Effect of oxygen on the inactivation of trypsin by ionising radiation.

30. Mee, L.K. (1958). 26th Ann. Rept. British Empire Cancer Society, p.318.

31. Sutton, H.C. (1952). Discussions Faraday Soc. p. 281.

32. Dale, W.M., Gray, L.H., and Meredith. W. (1949) Phil. Trans. Roy. Soc. London 242A, 33-62 Inactivation of an enzyme (carboxypeptidase) by X- and alpha- radiation.

33. Holmes, B.   (1950) Nature 165, 266-269.   Inactivation
    of ribonuclease in dilute aqueous solutions.

34. Hutchinson, F.   (1960) Radiat.   Res.   14, 721-731.   The
    effect of oxygen and some other gases on the radiation
    sensitivity of dry trypsin.

35. Adams, G.E., Redpath, J.L., Bisby, R.H., and Cundall,
    R.B.   (1972) Israel J.   Chem.   10, 1079-1093.   The use
    of free radical probes in the study of mechanisms of
    enzyme inactivation.

36. Powers, E.L.   (1972) Israel J.   Chem.   10, 1199-1211.
    The hydrated electron, the hydroxyl radical and hydrogen
    peroxide in radiation damage in cells.

37. Adams, G.E., Boag, J.W., and Michael, B.D.   (1966)
    Proc.   Roy.   Soc.   (London) A289, 321.   Transient
    species produced in irradiated water and aqueous
    solutions containing oxygen.

38. Cohen, G., and Heikkila, R.   (1974) J.   Biol.   Chem.
    249, 2447-2452.   Generation of hydrogen-peroxide,
    superoxide radical, and hydroxyl radical by
    6-hydroxydopamine, dialuric acid, and related cytotoxic
    agents.

39. Mc Cord, J.M.   and Fridovich, I.   (1973) Photochem.
    Photobiol.   17, 115-121.   Production of superoxide in
    photolyzed water demonstrated through the use of
    superoxide dismutase.

40. Revesz, L.   (1974).   In: 5th International Hyperbaric
    Congress (Vancouver, Canada).   Trapp, W., E.   Bannister,
    A.   Davison and P.   Trapp (eds.) Simon Fraser
    University.   Recovery process and the role of oxygen.

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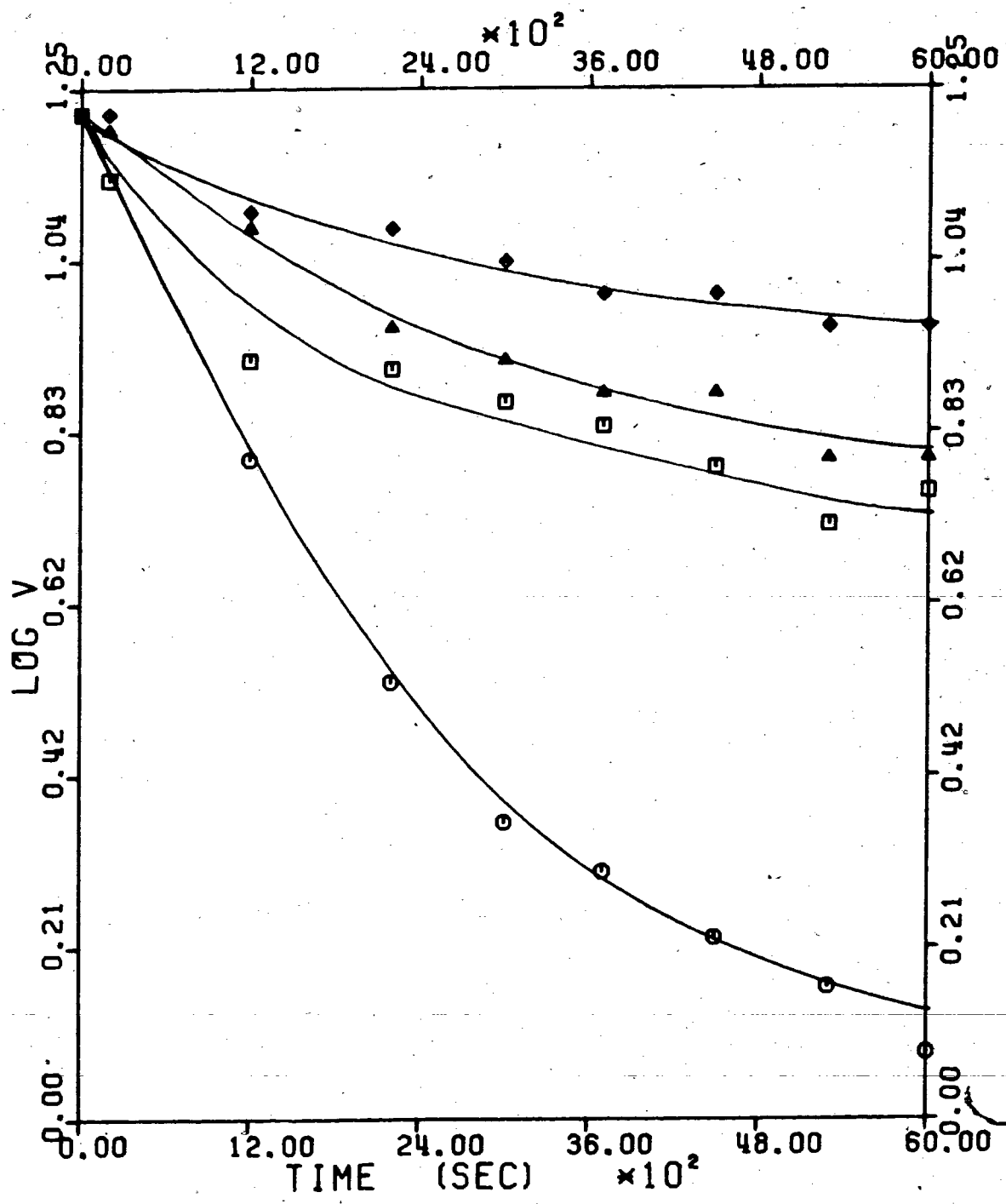
FIGURE 2. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE
DESTRUCTION OF CATALASE BY ASCORBATE IN THE PRESENCE OF
COPPER. Conditions were: 0.1 M phosphate buffer pH 7.0,
temperature 25 deg C, catalase concentration 10 uM,
ascorbate concentration 10 mM, copper(II) acetate
concentration 5 mM.

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.
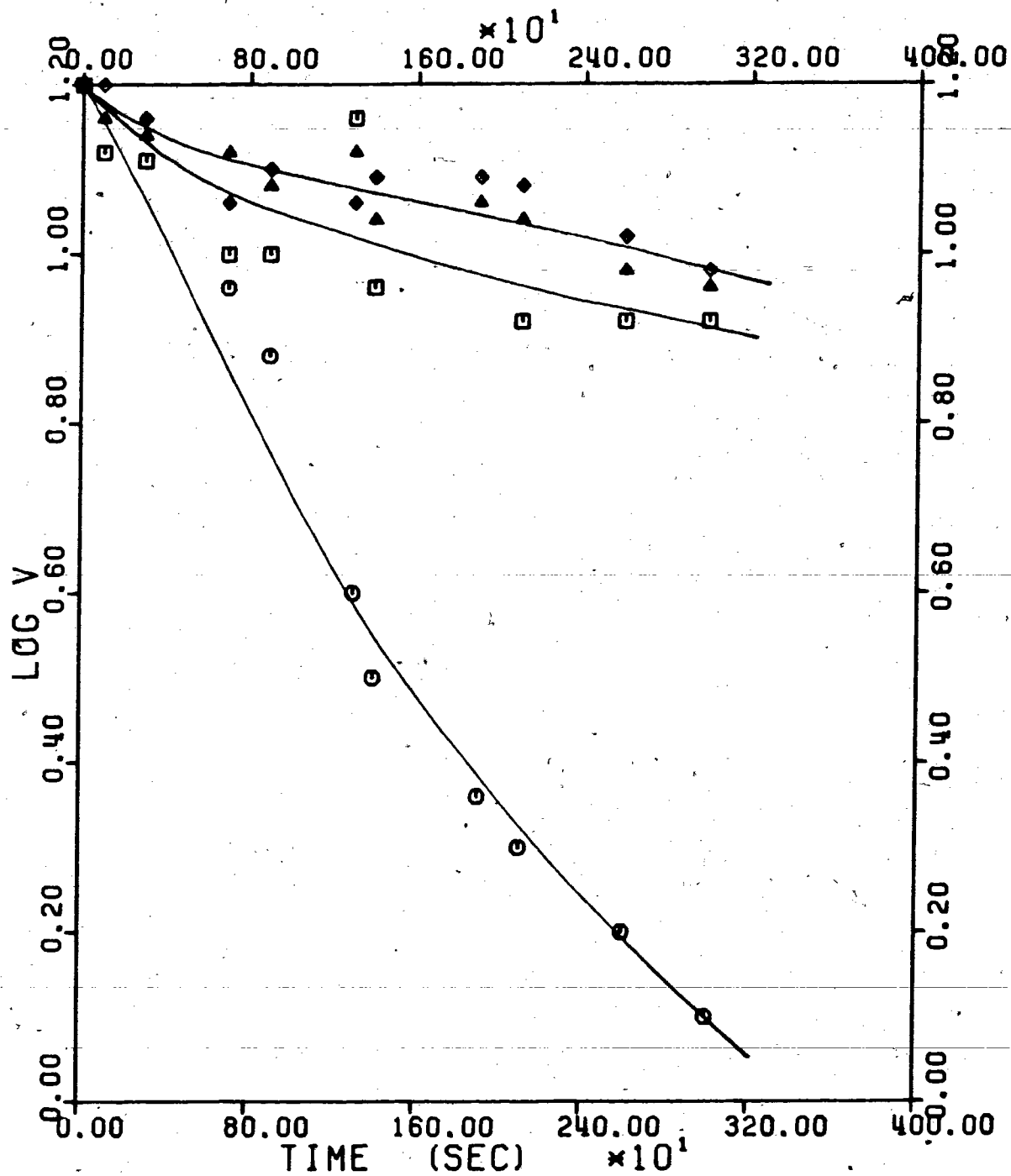
FIGURE 3. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE RATE
OF HAEMOLYSIS INDUCED IN WASHED ERYTHROCYTES BY THE PRESENCE
OF 5 nM DOPA. Conditions were: 0.01 M phosphate buffer pH
7.0 rendered isotonic by the addition of NaCl to 300 mOsM,
temperature 25 deg C, concentration of cells 5 % v/v.

Symbols

     Nitrogen   ⊕

     Air   ▣

     Oxygen   ▲

FIGURE 3. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE RATE
OF HAEMOLYSIS INDUCED IN WASHED ERYTHROCYTES BY THE PRESENCE
OF 5 nM DOPA.
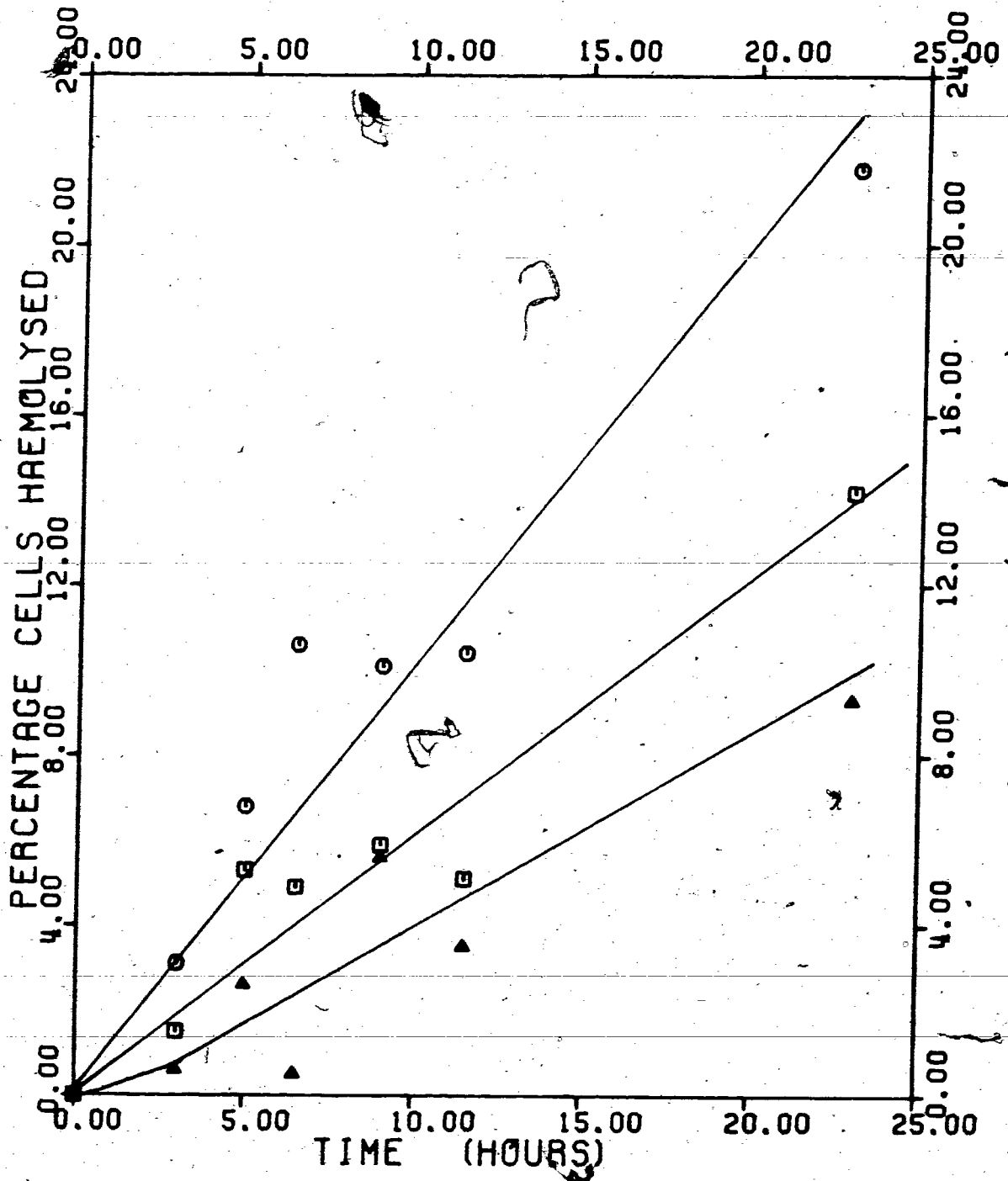
FIGURE 4. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE
FORMATION OF METHAEMOGLOBIN, INDUCED IN WASHED INTACT
ERYTHROCYTES BY THE PRESENCE 5 mM DOPA. Conditions were:
0.01 M phosphate buffer pH 7.0 rendered isotonic by the
addition of NaCl to 300 mOsM, temperature 25 deg C,
concentration of cells 5 % v/v.

Symbols

     Nitrogen   ◐

     Air   ▥

     Oxygen   ▲

FIGURE 4. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE
FORMATION OF METHAEMOGLOBIN, INDUCED IN WASHED INTACT
ERYTHROCYTES BY THE PRESENCE 5 mM DOPA.
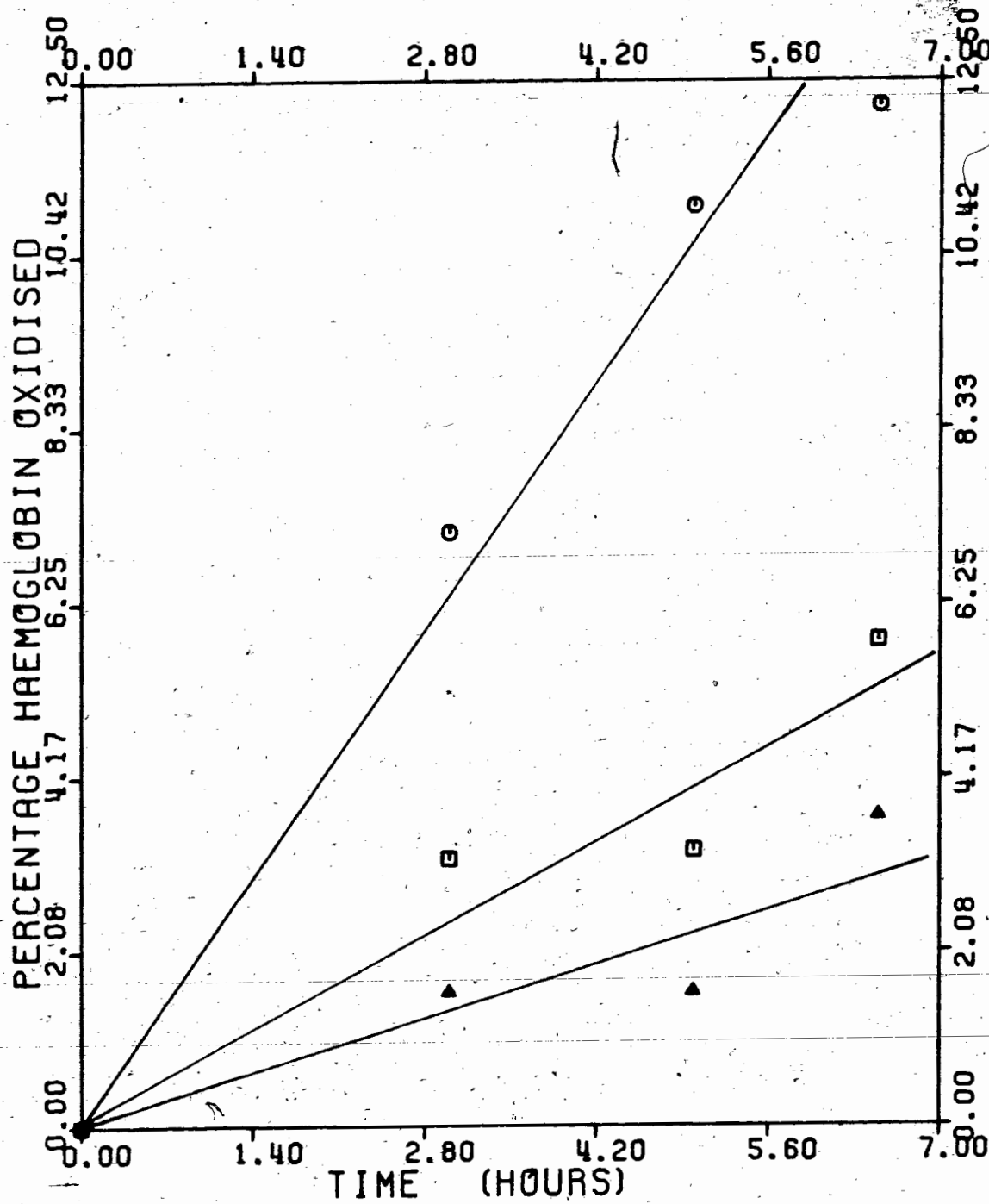
FIGURE 5. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE
DESTRUCTION OF CYTOCHROME C BY GAMMA-IRRADIATION.
Conditions were: 0.1 M phosphate buffer pH 7.0, temperature
23.5 deg C, cytochrome c concentration 30 uM.

Symbols

      Nitrogen   ⊕

      Air   ⊓

      Oxygen   △

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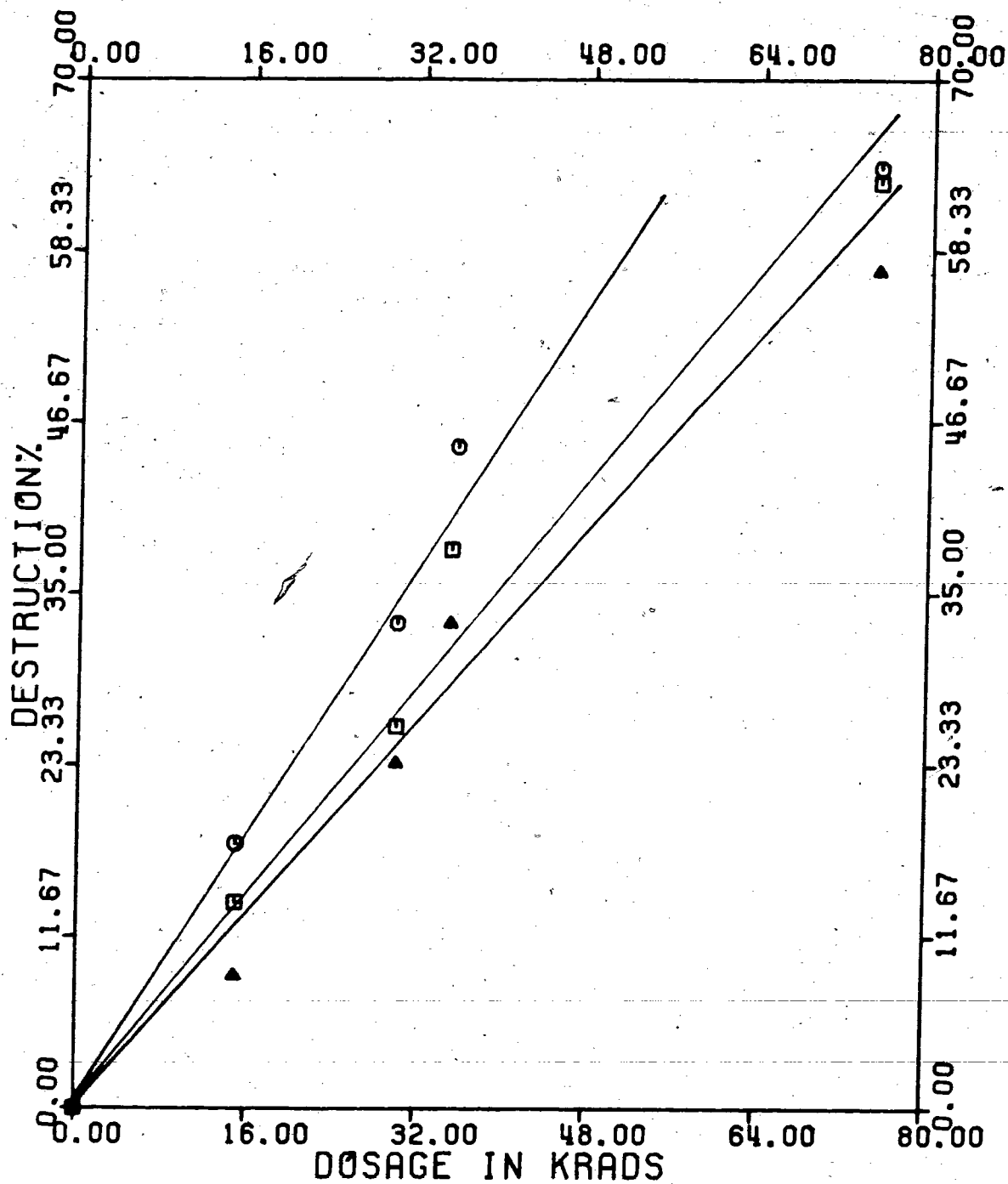
FIGURE 6. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE
DESTRUCTION OF THE PLASMA MEMBRANE OF RESEALED ERYTHROCYTE
GHOST CELLS BY GAMMA-IRRADIATION AT A RATE OF 0.5 KRAD/MIN.
Conditions were: 0.01 M phosphate buffer pH 7.4 rendered
isotonic by the addition of NaCl to 300 mOsM, temperature 25
deg C, concentration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

Symbols

Control	$\times$	$+$
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.
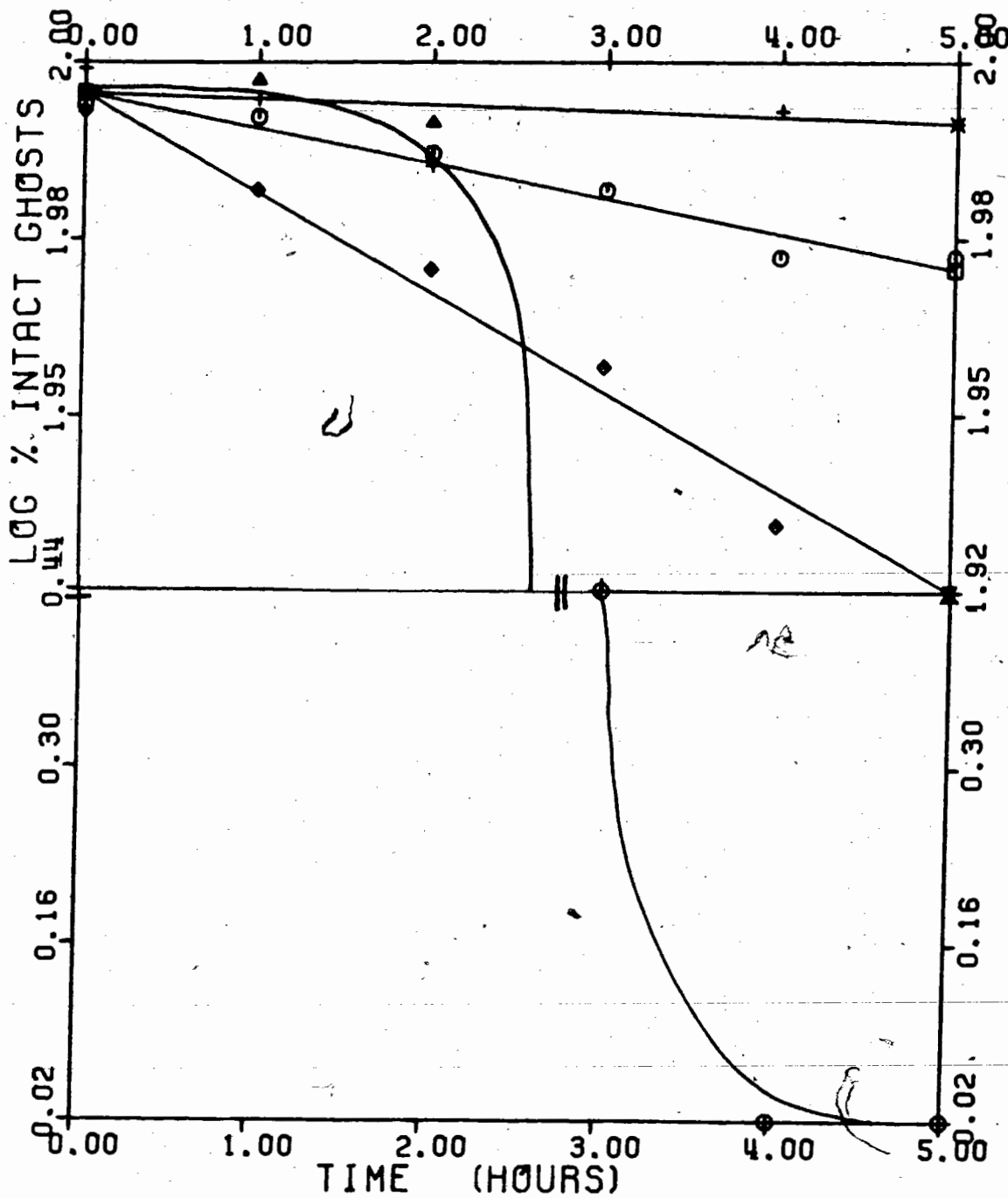
FIGURE 7. EFFECT OF THE CONCENTRATION OF OXYGEN ON THE
DESTRUCTION OF MEMBRANE-BOUND GLYCERALDEHYDe-3-PHOSPHATE
DEHYDROGENASE OF RESEALED ERYTHROCYTE GHOST CELLS BY
GAMMA-IRRADIATION AT A RATE OF 0.5 KRAD PER MIN. Conditions
were: 0.01 M phosphate buffer pH 7.4 rendered isotonic by
the addition of NaCl 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.
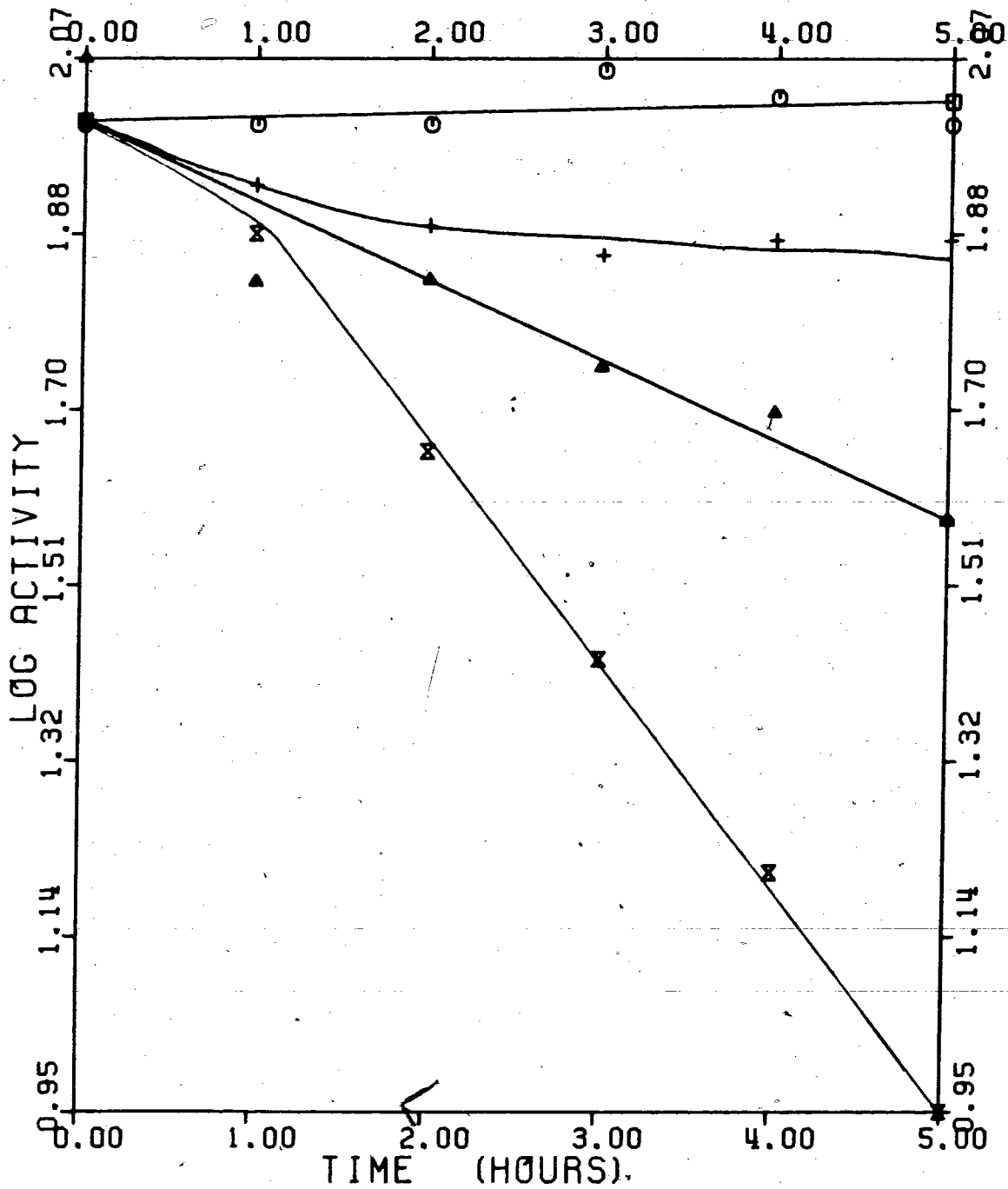
TABLE 1. EFFECTS OF OXYGEN CONCENTRATION ON THE RATE OF
DESTRUCTION OF CYTOCHROME C DURING GAMMA-IRRADIATION, IN THE
PRESENCE OF FORMATE, SUPEROXIDE DISMUTASE, OR LECITHIN.
Values given are uM cytochrome c destroyed per minute during
60.5 krad of radiation given over a period of one hour,
based on the 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.

SYSTEM	RATE OF DESTRUCTION OF CYTOCHROME $\underline{c}$ DURING IRRADIATION ($\mu M.min^{-1}$)		
	Oxygen	Air	N_2
Cytochrome $\underline{c}$	.0965 ± .012	.110 ± .014	.204 ± .026
Cytochrome $\underline{c}$ + superoxide dismutase	.147 ± .018	.127 ± .016	.134 ± .017
Cytochrome $\underline{c}$ in formate	.0379 ± .005	.0620 ± .008	.231 ± .029
Cytochrome $\underline{c}$ in formate + superoxide dismutase	.0302 ± .004	.0439 ± .006	.213 ± .027
Cytochrome $\underline{c}$ + lecithin	.105 ± .013	.111 ± .014	.177 ± .022

SECTION 2

ACTIONS OF GAMMA RADIATION ON RESEALED ERYTHROCYTE GHOSTS. A COMPARISON WITH INTACT ERYTHROCYTES AND A STUDY OF THE EFFECTS OF OXYGEN

by

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ABSTRACT

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,
Nenadovic 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, Taverna and Langdon 1973,
Zwaal, Roelofsen and Colley 1973, Tillmann, Cordua and

Schroter 1975, Rodan, Rodan 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), dithicthreitol (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:

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

% 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.

$$\% \text{ active G3PDH} = \frac{\text{G3PDH activity (+Triton) at any dose}}{\text{G3PDH activity (+Triton) at zero dose}} \times 100\%$$

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 x 10*19 ev.l-1min-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 "ANCOVA1" 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 comparson with unirradiated ghosts (Figure 2). The rate of inactivation of G3PDH was 22-fold greater in ghosts ($84.3 \times 10^{*-3}$ 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 crder cf

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 concentraticns 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 cxygen is

better illustrated by the observation that the aercbic 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-fcld.

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 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 unacceptible. 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 Hemmen, 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 .OH, O2- and H2O2 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 1979), 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:

$$L. + O_2 \longrightarrow LOO. \tag{1}$$

$$LOO. + LH \longrightarrow LOOH + L. \tag{2}$$

Chain branching:

$$LOOH \longrightarrow LO. + .OH \tag{3}$$

$$LO. + LH \longrightarrow LOH + L. \tag{4}$$

$$.OH + LH \longrightarrow H_2O + L. \tag{5}$$
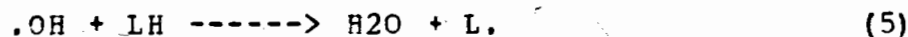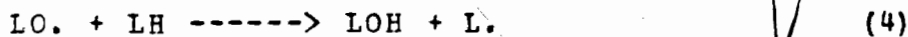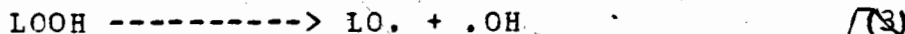
Since the rate of reaction (1) between the radical formed in the lipids and dissolved O_2 depends on the concentration of O_2 in the membrane, a high concentration of O_2 markedly increases the overall rate. In oxygen-saturated solutions, the concentration of O_2 in the membrane would be higher than in the aqueous phase, since the solubility of O_2 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 L. 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/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 1. EFFECTS OF CONCENTRATIONS OF OXYGEN ON RATES OF DAMAGE TO MEMBRANE-BOUND G3PDH AND MEMBRANE OF ERYTHROCYTES AND GHOSTS.

	rate of increase in permeability x 10*-3	rate of inactivation of G3PDH x 10*-3
unirradiated ghosts in buffers:		
air-saturated	0.97 +/-1.6	-4.11+/-6.01
oxygenated	1.66+/-2.26	0.97+/-1.48
irradiated ghosts in buffer:		
air-saturated	16.45+/-3.15	84.28+/-7.99
oxygenated	2.75+/-3.29 (initial)	119.5+/-16.5 (initial)
	986+/-223 (final)	229.8+/-11.6 (final)
nitrogen	5.79+/-0.67	55.5+/-7.62
erythrocytes irradiated in air-saturated buffer	-0.10+/-0.36	3.94+/-2.92

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.
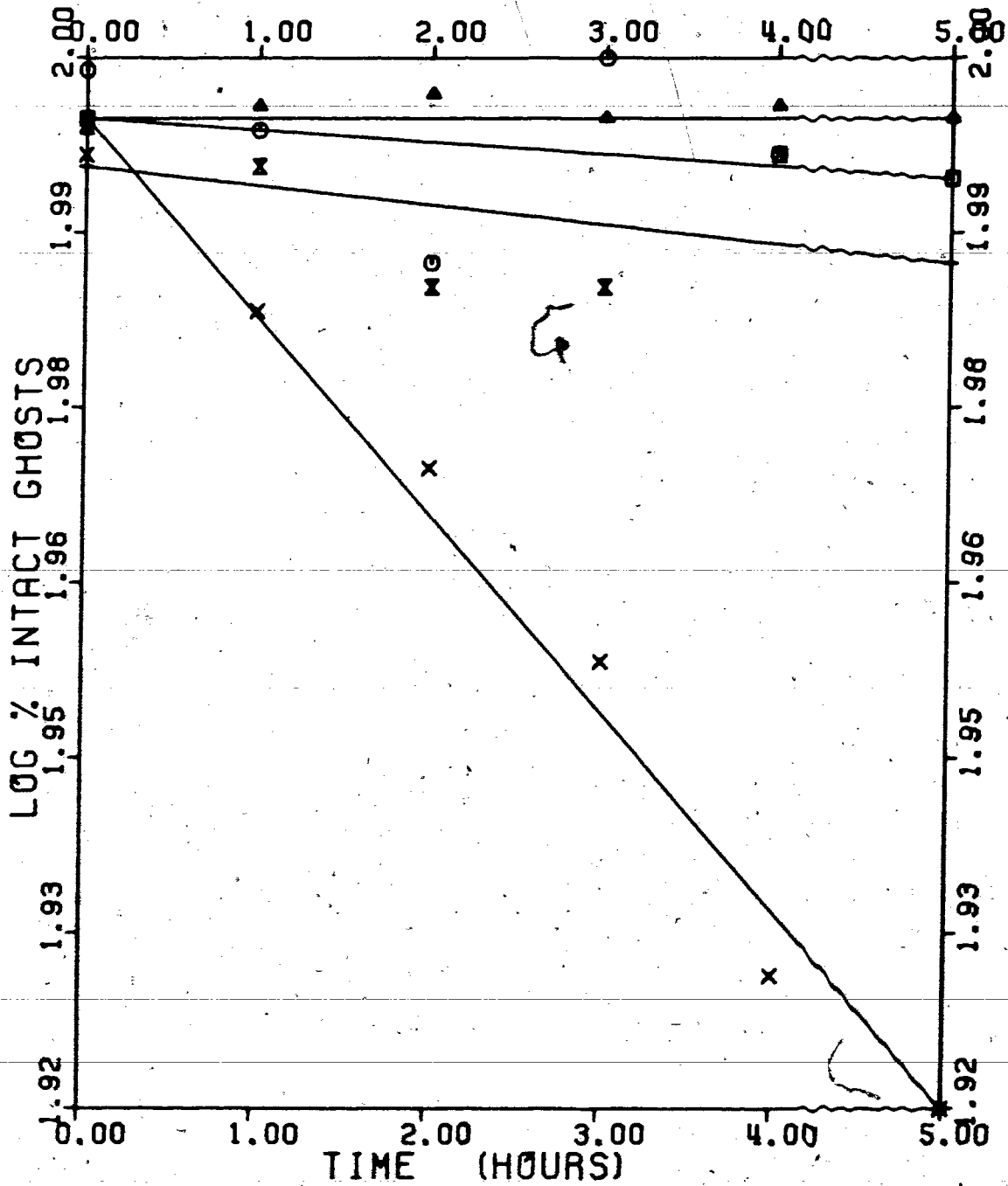
FIGURE 2. COMPARISON OF THE EXTENT OF RADIATION DAMAGE TO
ACTIVITY OF G3PDH OF ERYTHROCYTES AND GHOSTS IN THE PRESENCE
OF AIR. Log per cent G3PDH remaining active after each hour
of irradiation was plotted against time in hour. Conditions
were as described in Figure 1.

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.
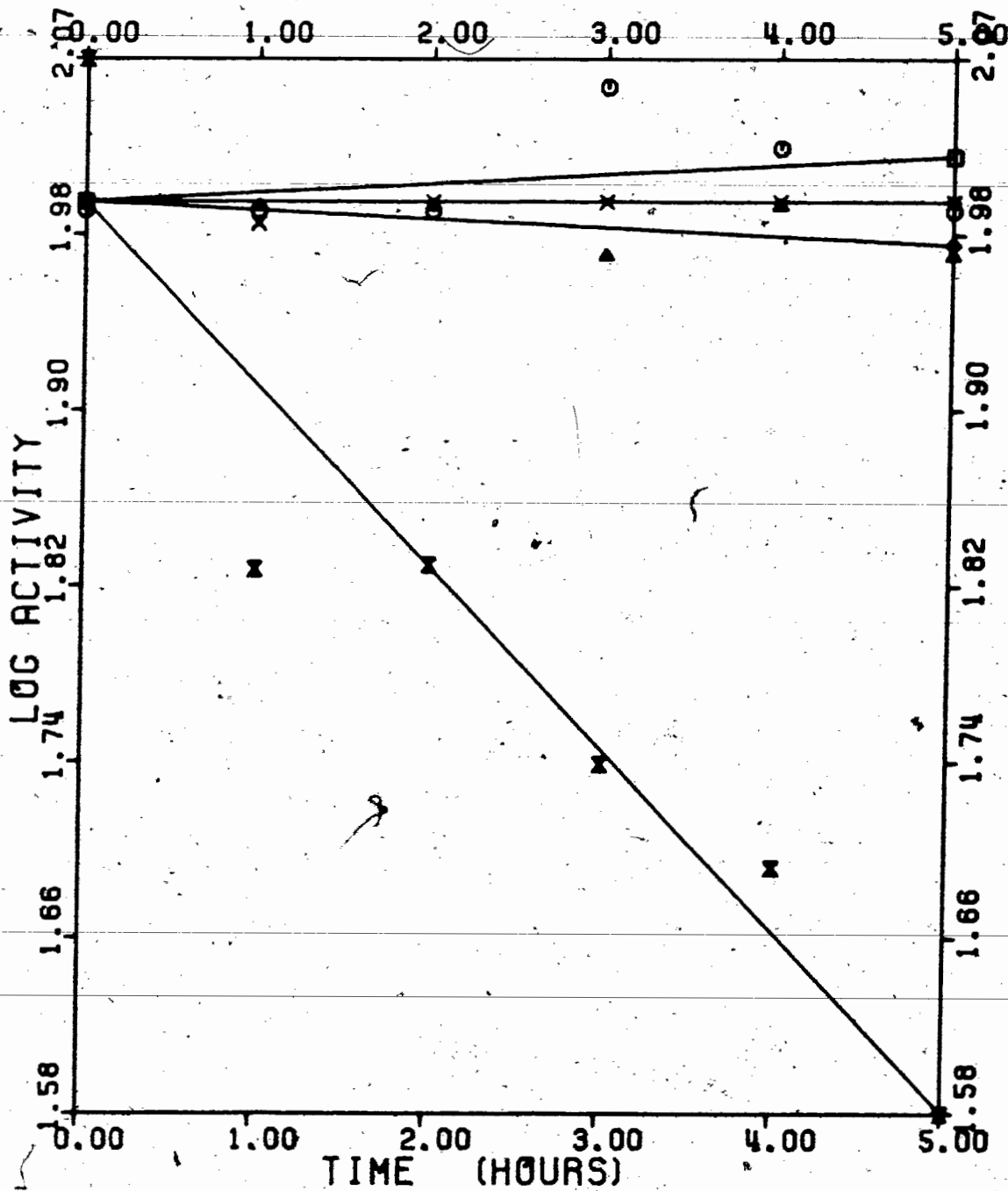
FIGURE 3. EXTENT OF RADIATION DAMAGE TO PERMEABILITY OF GHOSTS AT DIFFERENT CONCENTRATIONS OF OXYGEN. Log per cent ghosts remaining intact after each hour of irradiation was plotted against time in hour. Conditions were as describde in Figure 1. Unirradiated ghosts incubated at 25 deg C in air-saturated 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	①	Ⅲ
Air	◈	⊠
Oxygen	▲	⊕

FIGURE 3. EXTENT OF RADIATION DAMAGE TO PERMEABILITY OF
GHOSTS AT DIFFERENT CONCENTRATIONS OF OXYGEN.
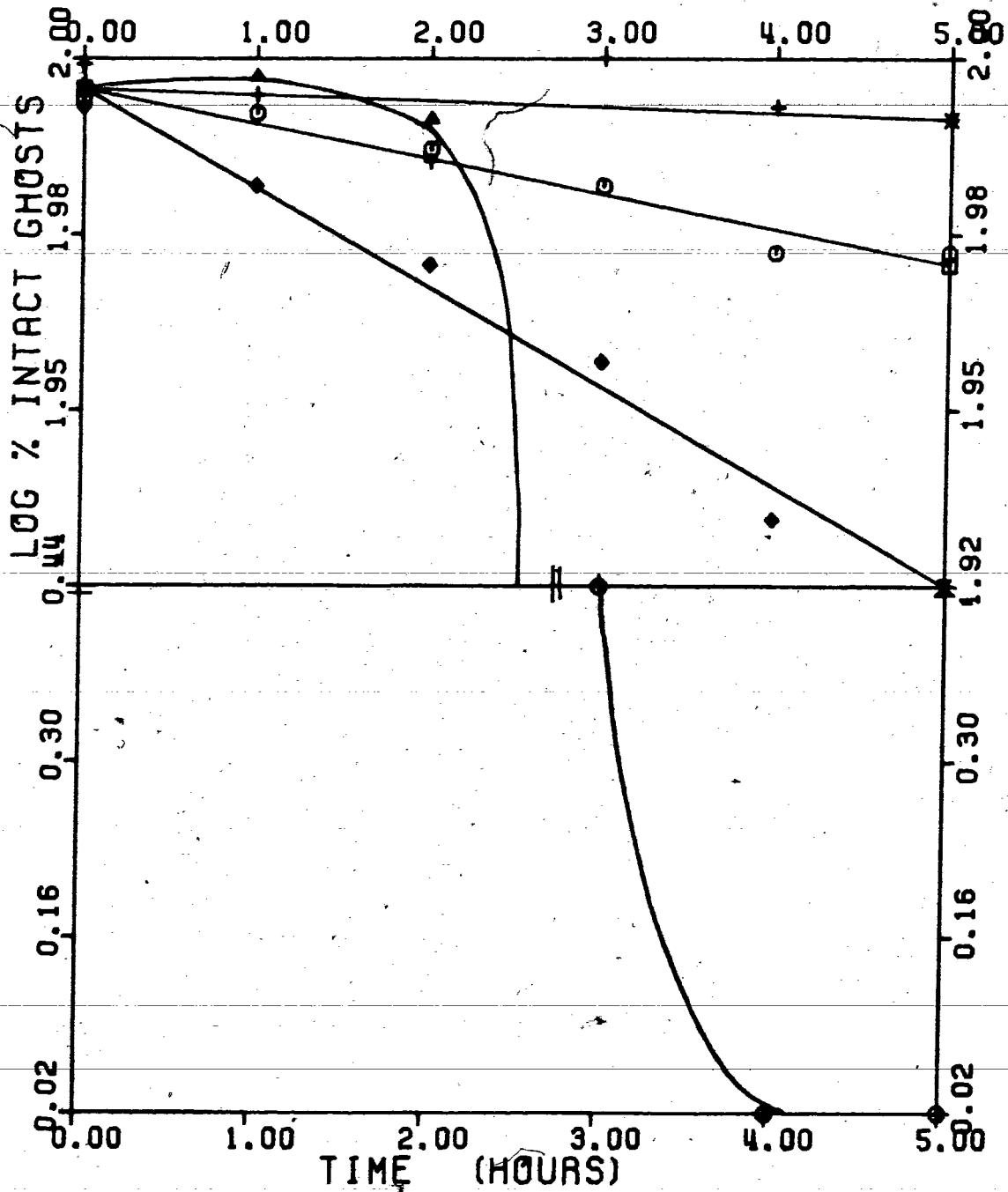
FIGURE 4.   EXTENT OF RADIATION DAMAGE TO ACTIVITY OF G3PDH

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   ✕

FIGURE 4. EXTENT OF RADIATION DAMAGE TO ACTIVITY OF G3PDH
OF GHOSTS AT DIFFERENT CONCENTRATIONS OF OXYGEN.
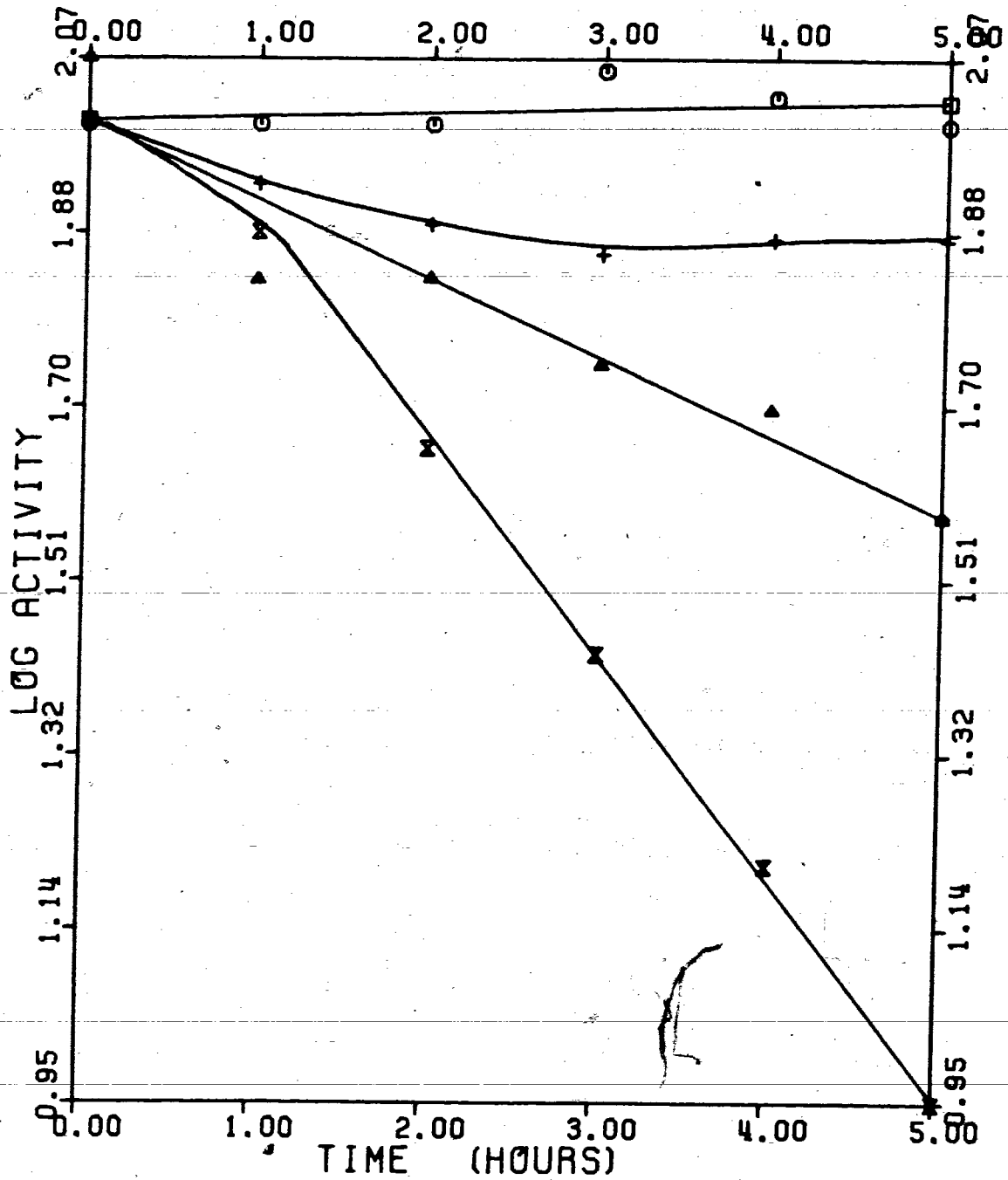
BIBLIOGRAPHY

Alper, T., 1971, Biophysical aspects of radiation quality, p.171. Vienna.

Alper, T., 1968, Nature (London), 217:862-863. Low oxygen enhancement ratios for radiosensitive bacterial strains, and the probable interaction of two types of primary lesion.

Allen, D.W., Jandl, J.H., 1961, J.Clin.Invest., 6:454-475. Oxidative hemolysis and precipitation of hemoglobin. II. Role of thiols in oxidant drug action.

Bland, J., Madden, P., and Herbert, E.J., 1975, Physiol. Chem. and Physics, 7:63-75. Effect of alpha-tocopherol on the rate of photohaemolysis in human erythrocyte.

Bland, J., Canfield, W., Kennedy, T., Vincent, J., Wells, R., 1978, Physiol.Chem. and Physics, 10:145-152. Effect of tocopherol on photoxidation rate of human erythrocyte membrane IN VITRO.

Bramley, T.A., Coleman, R., Finean, J.B., 1971, Biochim.Biophys.Acta, 241:752-769. Chemical, enzymological and permeability properties of erythrocyte ghosts prepared by hypotonic lysis in media of different osmolarities.

Bramley, T.A., Coleman, R., 1972, Biochim.Biophys.Acta, 290:219-228. Effects of inclusion of Ca2+, Mg2+, EDTA or EGTA during the preparation of erythrocyte ghosts by hypotonic haemolysis.

Buchanan, J.D., Armstrong, D.A., 1978, Int.J.Radiat.Biol., 33:410-419. The radiolysis of glyceraldehyde-3-phosphate dehydrogenase.

Davison,A.J., Kaminsky, L.S., 1974, Proc.5th.Int.Hyperbaric Congress (Vancouver), Trapp, W., Bannister, E., Davison, A., Trapp, P. (eds.), Simon Fraser Press. Involvement of oxygen radicals in radiation damage.

Davison, A., Popma, A., 1978, Int.Congr.Nutrition, 10:146. Effects of excess retinol, tocopherol, or ascorbate on lipid autoxidation, enzyme inactivation, and permeability of the plasma membrane of erythrocyte ghosts.

Demopoulos, H.B., 1973a, Fed.Proc., 32:1859-1861 The basis of free radical pathology.

Demopoulos, H.B., 1973b, Fed.Proc., 32:1903-1908. Control of free radicals in biologic systems.

Dodge, J.T., Mitchell, C., Hanahan, D.J., 1963, Arch.Bioch.Biophys., 100:119-130. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes.

Draganic, I.G., Draganic, Z.D., 1969a, J.Phys.Chem., 73:2571-2577. On the origin of primary hydrogen peroxide yield in the gamma radiolysis of water.

Draganic, I.G., Nenadovic, M.T., Draganic, Z.D., 1969b, J.Phys.Chem., 73:2564-2571. Radiolysis of HCOOH + O2 at pH 1.3-13 and the yields of primary products in gamma radiolysis of water.

Draganic, Z.D., Draganic, I.G., 1971, J.Phys.Chem., 75:3950-3957. Studies of formation of primary yields of hydrogen peroxide and molecular hydrogen in the gamma radiolysis of neutral aqueous solutions.

Draganic, Z.D., Draganic, I.G., 1973, J.Phys.Chem., 77:765-772. Studies on the formation of primary yields of hydroxyl radical and hydrated electron in the gamma radiolysis of water.

Duchon, G., Collier, B., 1971, J.Membr.Biol., 6:138-157. Enzyme activities of human erythrocyte ghosts : effects of various treatments.

Ewing, D., 1978, Radiat.Res., 73:121-136. Additive sensitization of bacterial spores by oxygen and p-nitroacetophenone.

Fee, J.A., Bergamini, R., Briggs, R., 1975, Arch.Biochm.Biophys., 169:160-167. Observations on the mechanism of the oxygen/dialuric acid-induced hemolysis of vitamin E-deficient rat red blood cells and the protective roles of catalase and superoxide dismutase.

Fee, J.A., Teitelbaum, H.D., 1972, Bioch.Biophys.Res.Commun., 49:150-157. Evidence that superoxide dismutase plays a role in protecting red blood cells against peroxidative hemolysis.

Hatefi, Y., Hanstein, W.G., 1970, Arch.Biochm.Bicphys., 138:78-86. Lipid oxidation in biological membranes. I. Lipid oxidation submitochondrial particles and microsomes induced by chaotropic agents.

Hanahan, D.J., 1973, Biochim.Biophys.Acta, 300:319-340. The erythrocyte membrane variability and membrane enzyme activity.

Hanstein, W.G., Hatefi, Y., 1970, Arch.Bioch.Biophys., 138:319-340. Lipid oxidation in biological membranes. II. Kinetics and mechanisms of lipid oxidation in submitochondrial particles.

Hoffman, J.F., 1958, J.Gen.Physiol., 42:9-28. Physiological characteristics of human red blood cell ghosts.

Howard-Flanders, P., 1960, Nature, 186:485-487. Effect of oxygen on the radiosensitivity cf bacteriophage in the presence of sulphydryl compounds.

Huyser, E.S., 1970, Free Radical Chain Reactions, Wiley-interscience.

Jacob, H.S., Jandl, J.H., 1962, J.Clin.Invest., 4:779-792. Effects of sulphydryl inhibition on red blood cells. I. Mechanism of hemolysis.

Jacob, H.S., Jandl, J.H., 1962, J.Clin.Invest., 7:1514-1523. Effects of sulphydryl inhibition on red blood cells. II. Studies IN VIVO.

Koch, C.J., Kruuv, J., Frey, H.E., 1973, Radiat.Res., 53:33-42. Variation in radiation response of mammalian cells as a function of oxygen tension.

Kong, S.K., M.sc. thesis, 1979, Simon Fraser University, British Columbia, Canada. The effects of free radicals and oxygen on the permeability and activity of glyceraldehyde-3-phosphate dehydrogenase of resealed erythrocyte ghosts.

Lin, W.S., Armstrong, D.A., 1978, Int.J.Radiat.Biol., 33:231-243. Effects of superoxide dismutase, dithicthreitol and formate ion on the inactivation of papain by hydroxyl and superoxide radicals in air-saturated solutions.

McCord, J.M., Fridovich. I., 1969, J.Biol.Chem., 244:6049-6055. Superoxide dismutase, an enzymatic function for erythrccuprein.

Michaels, H.B., Hunt, J.W., 1978, Radiat.Res., 74:23-34. A model for radiation damage in cells by direct effect and by indirect effect: a radiation chemistry approach.

Nicholls, P., 1972, Biochim.Biophys.Acta., 279:3C6-309. Contribution of catalase and glutathione peroxidase to red cell peroxide removal.

Petkau, A., Chelack, W.S., 1976, Biochim.Biophys.Acta., 433:445-456. Radioprotective effect of superoxide dismutase on model phospholipid membranes.

Petkau, A., Kelly, K., Chelack, W.S., Barefoot, C., 1976, Bioch.Bophys.Res.Comm., 70:452-458. Protective effect of superoxide dismutase on erythrocytes of X-irradiated mice.

Popma, M.A., M.sc. thesis, 1974, Simon Fraser University, British Columbia, Canada. The effects of retinol, alpha-tocopherol and ascorbic acid on the plasma membrane of erythrocyte ghosts.

Purdie, J.W., 1971, Radiat.Res., 48:474-483. Investigation of chain reactions and oxygen effects during radiolysis of peptide disulphide bonds using cysteine-glutathione disulphide as a model.

Quintiliani, M., Badiello, R., Tamba, M., Esfandi, A., Gorin, G., 1977, Int.J.Radiat.Biol., 32:195-202. Radiolysis of glutathione in oxygen-containing solutions of pH 7.

Raleigh, J.A., Kremers, W., Gaboury, B., 1977, Int.J.Radiat.Biol., 31:203-213. Dose-rate and oxygen effects in models of lipid membranes: linoleic acid.

Rapoport, S.M., Muller, M., 1974, p.167-180, Catalase and Glutathione peroxidase, "Cellular and Molecular Biology of Erythrocytes" edited by Yoshikawa, H., and Rapoport, S.M., University Park Press, London.

Rodan, S.R., Rodan, G.A., Sha'afi, R.I., 1976, Biochim.Biophys.Acta, 428:509-515. Demonstration of adenylate cyclase activity in human red blood cell ghosts.

Schrier, S.L., 1966, Am.J.Physiol., 210:139-145. Organisation of enzymes in human erythrocyte membranes.

Schrier, S.L., 1963, J.Clin.Invest., 42:756-766. Studies of the metabolism of human erythrocyte membranes.

Schwoch, G., Passow, H., 1973, Molecular and Cellular Biochemistry, 2:197-218. Preparation and properties of human erythrocyte ghosts.

Sokal, R.R., Rohlf, F.J., 1969, Biometry, the principles and practice of statistics in biological research, Freeman and Co., p.448-458.

Staros, J.V., Haley, B.E., Richards, F.M., 1974,
J.Biol.Chem., 249:5004-5007 Human erythrocytes and resealed
ghosts: a comparison of membrane topology.

Steck, T.L., Kant, J.A., 1974, Methods Enzymol., 31:172-180.
Preparation of impermeable ghosts and inside-out vesicles
from human erythrocyte membranes.

Stocks, J., Dormandy, T.L., 1971, Brit.J.Haematol.,
20:95-111. The autoxidation of human red cell lipids
induced by H2O2.

Sutton, H.C., Roberts, P.B., Winterbourn, C.C., 1976,
Biochem.J., 155:503-510. The rate of reaction of superoxide
radical ion with oxyhaemoglobin and methaemoglobin.

Tappel, A.L., 1973, Fed.Proc., 32:1870-1874. Lipid
peroxidation damage to cell components.

Taverna, R.D., Langdon, R.G., 1973, Biochim.Biophys.Acta,
298:422-428. Glucose transport in white erythrocyte ghosts
and membrane-derived vesicles.

Tillmann, W., Cordua, A., Schroter, W., 1975,
Biochim.Biophys.Acta, 382:157-171. Organisation of enzymes
of glycolysis and of glutathione metabolism in human red
cell membranes.

Van Hemmen, J.J., Meuling, W.J.A., Bleichrodt, J.F., 1978,
Radiat.Res. 75:410-423. Effect of oxygen on inactivation
of biologically active DNA by gamma rays IN VITRO :
influence of metalloporphyrins and enzymatic DNA repair.

Wills, E.D., Wilkinson, A.E., 1967, Radiat.Res., 31:732-747.
The effect of irradiation on lipid peroxide formation in
subcellular fractions.

Wills, E.D., Rotblat, J., 1965, Int.J.Radiat.Biol.,
8:551-567. The formation of peroxides in tissue lipids and
unsaturated fatty acids by irradiation.

Woodward, C.B., Zwaal, R.F.A., 1972, Biochim.Biophys.Acta., 274:272-278. The lytic behaviour of pure phophoslipases A2 and C towards osmotically swollen erythrocytes and resealed ghosts.

Yuhas, J.M., Li, A.P., 1978, Radiat.Res., 75:563-572. In vitro studies on the radioresistance of oxic and hypoxic cells in the presence of both radioprotective and radiosensitizing drugs.

Yung, C.Y., Carlson, L.M., Balzer, C.J., 1972, Biochim.Biophys.Acta, Characteristics of the permeability barrier of human erythrocyte ghosts to nonelectrolytes.

Zimmermann, R., Flohe, L., Weser, U., Hartmann, H.J., 1973, FEBS Letter, 29:117-120. Inhibition of of lipid peroxidation in isolated inner membrane of rat liver mitochondria by superoxide dismutase.

Zwaal, R.F.A., Roelofsen, B., Colley, C.M., 1973, Biochim.Biophys.Acta, 300:159-182 Localisation of red cell membrane constituents.

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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ABSTRACT

The relative effectiveness of oxidizing (.OH, H2O2), ambivalent (O2-) and the reducing free radicals: (e- and CO2-), in causing damage to membranes and membrane-bound G3PDH of resealed erythrocyte ghosts has been determined. Rates of damage to membrane-bound G3PDH (R(enz)) were measured and the rates of damage to membranes (R(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 appropiate mixtures of scavengers such as catalase, superoxide dismutase and formate. .OH, O2- and H2O2 were approximately equally effective in inactivating membrane-bound G3PDH while, e- and CO2- were the least effective. R(enz) for O2- and H2O2 were 10-fold and for .OH 15-fold, that of e-. R(mb) values were quite similar for e- and H2O2; about twice that of O2- while that for .OH is 3-fold that of O2-. Hence, with respect to R(mb): .OH > e- = H2O2 > O2-, and with respect to R(enz): OH > O2- = H2O2 >> 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*10
M-1.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

included: 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 O2- is catalysed by metal complexes (Svingen 1978), and both O2- 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, Kellog 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 H2O2 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 O2-, H2O2 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 O2 produces the following primary free radicals (Draganic and Draganic 1971, Swallow 1973).

$$H2O \ \text{------>} \ e-, \ H., \ .OH, \ H2O2 \hspace{3cm} (1)$$
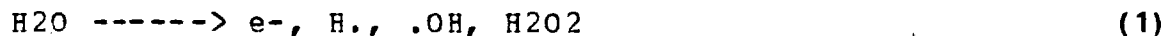
and their initial yields are : $G(e-)=2.8$, $G(H.)=0.6$, $G(.OH)=2.8$ and $G(H2O2)=0.6$ (Draganic et al. 1969a, 1969b, 1971, 1972, 1973). G is the number of molecules or ions formed per 100 ev. energy absorbed.

In the presence of oxygen, the following subsequent
reactions occur, resulting in a variety of secondary
radicals,

$$O_2 + e^- ---> O_2^- \qquad k = 1.88 \times 10^{*}10 \ M^{-1}.s^{-1} \quad (2)$$

$$O_2 + H ---> HO_2 \qquad k = 1.90 \times 10^{*}10 \ M^{-1}.s^{-1} \quad (3)$$

$$HO_2 ---> H^+ + O_2^- \qquad pK = 4.8 \ (Behar \ 1970) \qquad (4)$$

$$HCOO^- + .OH ---> COO^- + H_2O \qquad k = 2.5 \times 10^{*}9 \ M^{-1}.s^{-1} \quad (5)$$

$$COO^- + O_2 ---> CO_2 + O_2^- \qquad k = 2.4 \times 10^{*}9 \ M^{-1}.s^{-1} \quad (6)$$

The above rate constants were taken from Anbar and Neta
(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 cr 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), dithicthreitol (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 cf 20 ug/ml (i.e. 20.4 units
superoxide dismutase and 50 units catalase per ml solution)
in the buffer soluticns. 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 .H produced in (1) react with O2 readily, and are converted to O2-. by the reactions (2) and (3). Hence the irradiation of air-saturated solution will produce an amount of O2- equal to that of e- and .H Catalase was used as scavenger for H2O2. It catalyses the decomposition cf H2O2 as in (7). Formate is an effective scavenger of .OH (Draganic 1969b) and it is used in our study because of the increase in yield of O2- due to reaction (6). Thus, irradiation of air-saturated aqueous solution containing 10 millimolar formate and catalase (50 units/ml) will produce predominantly O2-. The total yield of O2- will be given by

$$G(O2-) = G(e-) + G(H.) + G(.OH) = 6.2$$

$$H2O2 + H2O2 \longrightarrow 2H2O + O2 \qquad k= 3.4 \times 10*7 \ M-1.s-1 \quad (7)$$
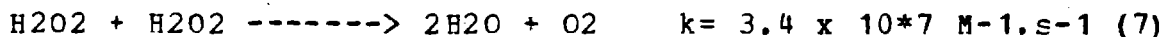
(Herbert and Pinsent 1948)

2.3.2. Production of H_2O_2

If superoxide dismutase (20.4 units/ml) was used instead of catalase in the above solution, O_2- produced will be converted to H_2O_2 according to (8).

$$2H+ + O_2- + O_2- -----> H_2O_2 + O_2 \qquad k = 2 \times 10*9 \ M-1.s-1 \ (8)$$
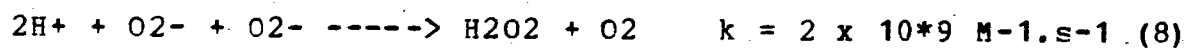
(Rabani, Klug and Fridovich 1972)

Therefore $G(H_2O_2) = G(H_2O_2) + 0.5 \times G(O_2-) = 0.6 + 3.1 = 3.7$

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

$$H + HCOO- -----> H_2 + COO- \qquad k = 2.5 \times 10*8 \ M-1.s-1 \ (9)$$

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), CO2- (G=3.4) and H2O2 (G=0.6).

2.3.4. Production of .OH

.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(mb) and R(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 O2-, H2O2, and e- plus CO2- 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(mb). Similarly, R(enz) is calculated for each kind of radical. The R values are listed in Table 2. R(mb) and R(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

$$G \cdot f \cdot r = D \qquad\qquad (10)$$

$$f \cdot r = D/G \qquad\qquad (11)$$

$$\text{since } R = D/G$$

$$\text{therefore } R = f \cdot r \qquad\qquad (12)$$

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 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 .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. .OH, O2- and H2O2 were about equally effective in inactivating membrane-bound G3PDH while e- and CO2- were the least effective. R(enz) for O2- and H2O2 were 12-fold and for .OH was 17-fold that of e-. R(mb) of e- and H2O2 were about 1.5-fold and of .OH 3-fold of that of O2-. 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(mb): .OH > e- = H2O2 > O2- and in order of decreasing R(enz): .OH >> O2- = H2O2 >> e-. Thus .OH was the most powerful radical in causing damage to both membrane and membrane-bound G3PDH.

3.3. Comparison of reagent H2O2 with H2O2 produced by radiation

Reagent H2O2 was added to a ghost suspension to a final concentration of 120 micromolar. Comparison of the damage due to reagent H2O2 with that due to H2O2 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 H_2O_2, it is obvious that radiolytically produced H_2O_2 is far more destructive.

4. Discussion.

Each of the 4 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 O_2^-
and H_2O_2 formed on the outside or that diffusing to the
outside 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 H_2O_2 (established in our experiments) and O_2^- (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

inhibit lysis of erythrocyte vesicles by O2- 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, O2- is

the least effective in causing lysis of the ghost membranes.

A much more effective damage to erythrocyte vesicles by O2-

was reported by Lynch (1978b). However, the presence of

complexed metals in their reaction medium may have catalysed

the lipid peroxidation by O2- (Svingen 1978) as well as by

.OH formed in the xanthine-xanthine oxidase system (Buettner

1978). The mechanism by which O2- increases permeability is

not established in the present study. It may be that O2-

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 O2- (Svingen 1978).

In view of the residual content of resealed ghosts, reaction of O2- with double bonds of lipids may be possible. A more plausible mechanism is the reaction of O2- 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 O2- 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 O2-. 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-.

$$E-SH + H^+ + O2- \longrightarrow E-S. + H2O2 \tag{13}$$

$$E-S. + O2 \longrightarrow E-SOO. \tag{14}$$

$$E-SOO. + O2- + H^+ \longrightarrow E-SOOH + O2 \tag{15}$$

$$E\text{-SOOH} + H_2O \longrightarrow E\text{-SOH} + H_2O_2 \qquad (16)$$

$$E\text{-SOH} + O_2^- + H^+ \longrightarrow E\text{-SO.} + H_2O_2 \qquad (17)$$

$$E\text{-SO.} \longrightarrow \text{inactive enzyme} \qquad (18)$$
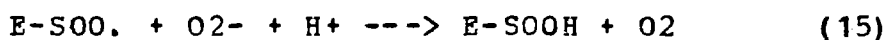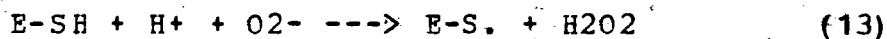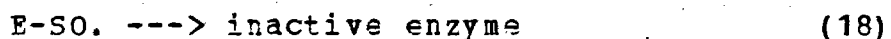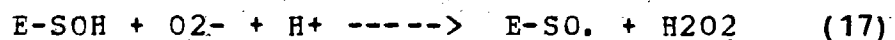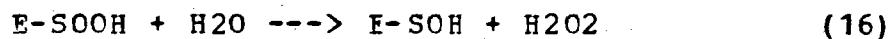
In spite of the facts that the amount of O_2^- produced in the bulk of the medium is significantly greater than that produced inside the ghosts, that it is hydrophilic, and that residual superoxide dismutase is present in the ghosts, O_2^- is effective in inactivating G3PDH for three reasons. Firstly, resealed ghosts are permeable to O_2^- (Lynch and Fridovich 1978a). Thus O_2^- produced outside ghosts can react with G3PDH on the cytoplasmic side. This permeability of membranes to O_2^- implies existance of anion channels because the negative charge on O_2^- would otherwise hinder its diffusion through the lipid bilayers. Secondly, its low reactivity towards membranes allowed most of O_2^- 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 O_2^-. 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 cf O2- on the cytoplasmic side of the membrane. However, C2- reaching an element of volume adjacent to the cytcplasmic side cf 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 concentraticn of dismutase in the element of volume adjacent to the internal surface of the membrane where G3PDH is located. Therefore the small amcunt of residual dismutase would not affect the R(mb) or R(enz) of O2- significantly.

4.2. Effects of hydrated electrons

Prom our data, we cannot separate the effects cf e- and CO2- since they were produced together. However, most cf 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 cf 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, Simic and Hayon 1970, Faraggi and Bettleheim 1977, Flossmann and Westhof 1978, Mittal and Hayon 1974, Rustgi, Joshi and Riesz 1977) leading to peptide, breakage and release of malonaldehydes (Tappel and Adhikari 1975).

$$-NH-CHR-C=O + e- \quad \text{-----}> \quad -NH-CHR-\overset{\bullet}{C}-O^- \qquad (19)$$

$$-NH-CHR-\overset{|}{C}-O^- + H+ \quad \text{-----}> \quad -NH2 + CHR=C-O^- \qquad (20)$$

$$CHR=\overset{|}{C}-O^- \quad <\text{-----}> \quad \overset{\bullet}{C}HR-\overset{|}{C}=O \qquad (21)$$
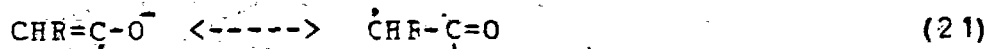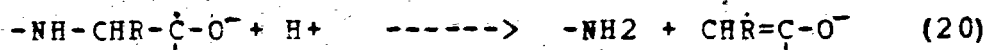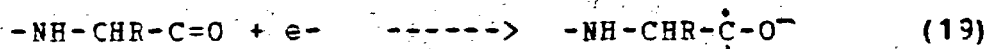
4.3. Effects of H2O2

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.

$$E-SH + H2O2 \text{----}> E-SOH + H2O \qquad (22)$$

$$E\text{-}SOH + HS\text{-}D\text{-}SH \longrightarrow E\text{-}SS\text{-}D\text{-}SH + H2O \qquad (23)$$

$$E\text{-}SS\text{-}D\text{-}SH \longrightarrow E\text{-}SH + DS2 \qquad (24)$$
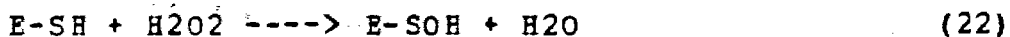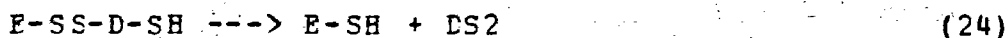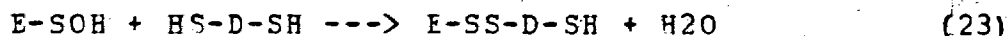
However, our data revealed only the additional irreversible inactivation because incubation with DTT did not restore enzyme activity. Reversible damage, if present, would not have been detected in the current study since DTT was routinely present in the assay medium. The possible contribution of .OH and O2- in the presence of both formate and superoxide dismutase is negligible due to the effectiveness of these scavengers. Singlet O2 may however be involved as it is thermodynamically feasible to produce singlet O2 via O2- 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

interaction of H_2O_2 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 H_2O_2 produced in the bulk of the aqueous medium. The R(mb) and R(enz) of H_2O_2 are the sums of damage due to H_2O_2 and the reactive species.

The difference in reactivity towards cellular constituents between reagent H_2O_2, and H_2O_2 produced by radiation was observed by Klebanoff (1958). It was found that much higher concentrations of reagent H_2O_2 had to be used to produce effects equal in magnitude to the effects of radiation. Alder (1963) found that H_2O_2 which had been generated enzymically, produced damage to cells comparable to that of H_2O_2 produced by radiation. This suggests that a continuous low level of H_2O_2 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(mb) for .OH could be explained by the high
reactivity of .OH towards phosphatidyl choline (rate
constant is 5.1x10*8 M-1.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.

PH + .OH -------> R. + H2O   (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 suprisingly

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, of 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 10mM. 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 1. EFFECTS CF FREE RADICALS ON PERMEABILITY AND
ACTIVITY OF MEMBRANE-BCUND G3PDH.

scavengers	radicals produced				rate of increase in permeability x $10*-3$	rate of inactivation of G3PDH x $10*-3$
	H_2O_2	O_2-	$e-$	CO_2-		
formate dismutase	3.7				3.5+/-1.6	29.74+/-3.9
formate catalase		6.2			3.93+/-1.07	53.93+/-8.76
N2 formate	0.6		2.8	3.4	5.45+/-1.33	6.40+/-2.27
catalase dismutase	2.8				5.05+/-1.42	32.47+/-3.68

TABLE 2.   THE EFFECTIVENESS OF H2O2, O2-, e- AND .OH IN

CAUSING DAMAGE TO PLASMA MEMBRANE AND MEMBRANE-BCUND G3PDH.

R(mb) and R(enz) were obtained by dividing the rates in

Table 1 by the yield of the corresponding free radical in

molecules cr ions per 100 ev.   Conditions were as described

in Table 1.

TABLE 2.   THE EFFECTIVENESS OF H_2O_2, O_2-, $e-$ AND $.OH$ IN
CAUSING DAMAGE TO PLASMA MEMBRANE AND MEMBRANE-BOUND G3PDH.

radical	R(mb) x 10*-3	R(enz) x 10*-3
H_2O_2	0.95+/-0.47	8.04+/-1.38
O_2-	0.63+/-0.2	8.69+/-1.76
$e- + CO_2-$	>0.79+/-0.22	>0.25+/-0.1
	<0.97+/-0.27	<1.14+/-0.45
$.OH$	1.80+/-0.58	11.60+/-1.78

FIGURE 1. COMPARISON OF THE EFFECTS OF O2-, H2O2, .OH AND
e- ON PERMEABILITY OF PLASMA MEMBRANES OF GHOSTS. Log per
cent 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   X

     O2-   *

     H2O2   ⊕

     H2O2 + e- + CO2-   △

FIGURE 1.   COMPARISON OF THE EFFECTS OF O2-, H2O2, .OH AND
e- ON PERMEABILITY OF PLASMA MEMBRANES OF GHOSTS.
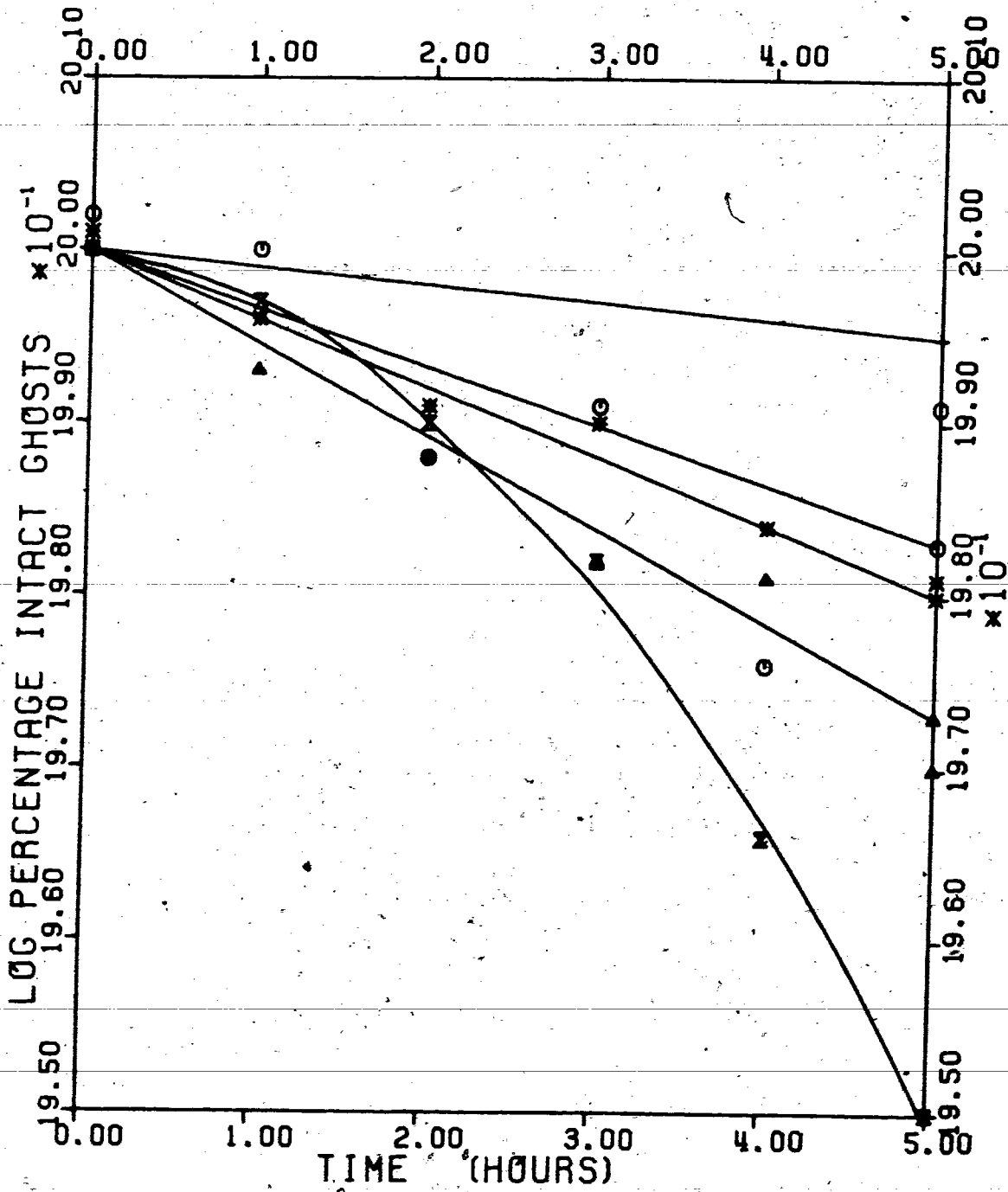
FIGURE 2. COMPARISON OF THE EFFECTS OF O2-, H2O2, .OH AND e- ON ACTIVITY OF MEMBRANE-BOUND G3PDH OF GHOSTS. Log per cent activity remaining after each hour of irradiation were plotted against time in hour. Conditions were as described in Figure 1.

Symbols

Control	+
.OH	X
O2-	*
H2O2	⊕
H2O2 + e- + CO2-	△

FIGURE 2. COMPARISON OF THE EFFECTS OF O2-, H2O2, .OH AND
e- ON ACTIVITY OF MEMBRANE-BOUND G3PDH OF GHOSTS.

FIGURE 3. COMPARISON CF THE EFFECT OF REAGENT H_2C_2 AND
RADIATION INDUCED H_2C_2 ON PERMEABILITY OF MEMBRANES. Log
per cent ghosts remaining intact after each hour cf
irradiation were plotted against time in hour. Conditions
were as described in Figure 1.

Symbols

      Control   +

      Radiation-induced H_2O_2 ✳
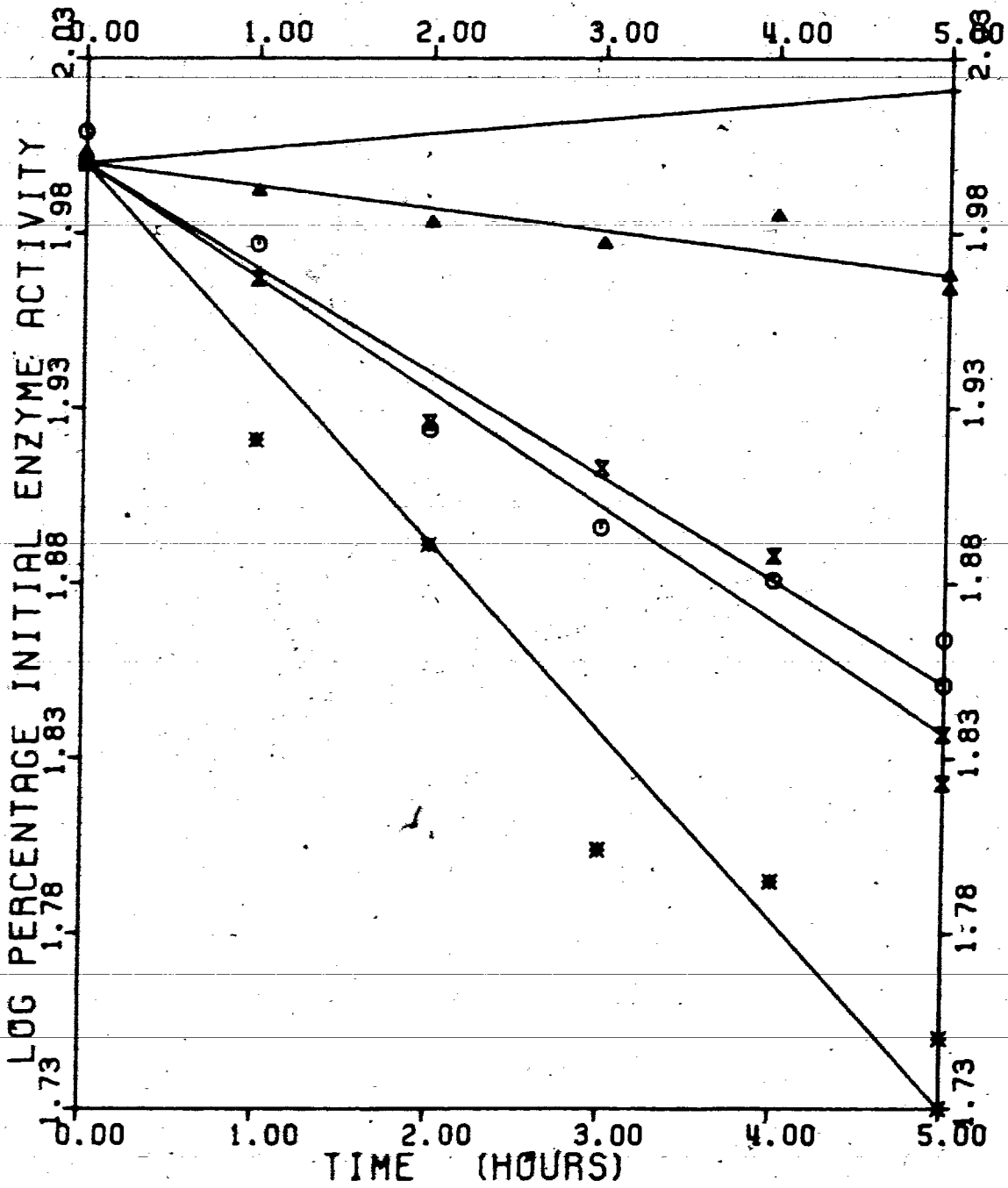
      Reagent H_2O_2 Ⅲ

FIGURE 3. COMPARISON OF THE EFFECT OF REAGENT H2O2 AND
RADIATION INDUCED H2O2 ON PERMEABILITY OF MEMBRANES.

FIGURE 4. COMPARISON OF THE EFFECT OF REAGENT H_2C_2 AND
RADIATION INDUCED H_2C_2 ON ACTIVITY OF MEMBRANE-BCUND G3PDH.
Log per cent activity remaining after each hour cf
irradiation were plotted against time in hour. Conditions
were as described in Figure 1.

Symbols

> Control $+$
> Radiation-induced H_2O_2 $\oplus$
> Reagent H_2O_2 $\boxdot$
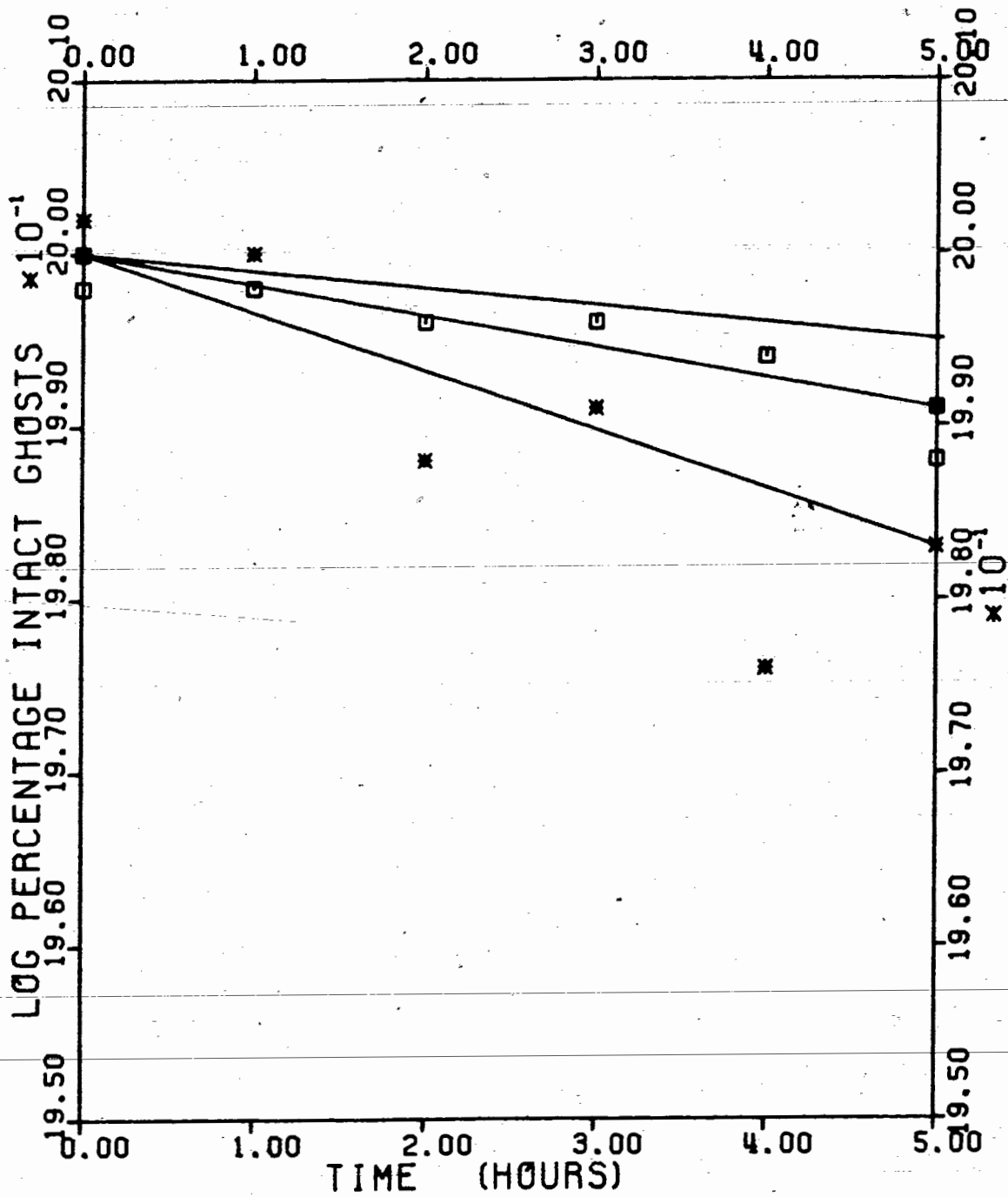
FIGURE 4. COMPARISON OF THE EFFECT OF REAGENT H2O2 AND
RADIATION INDUCED H2O2 ON ACTIVITY OF MEMBRANE-BOUND G3PDH.
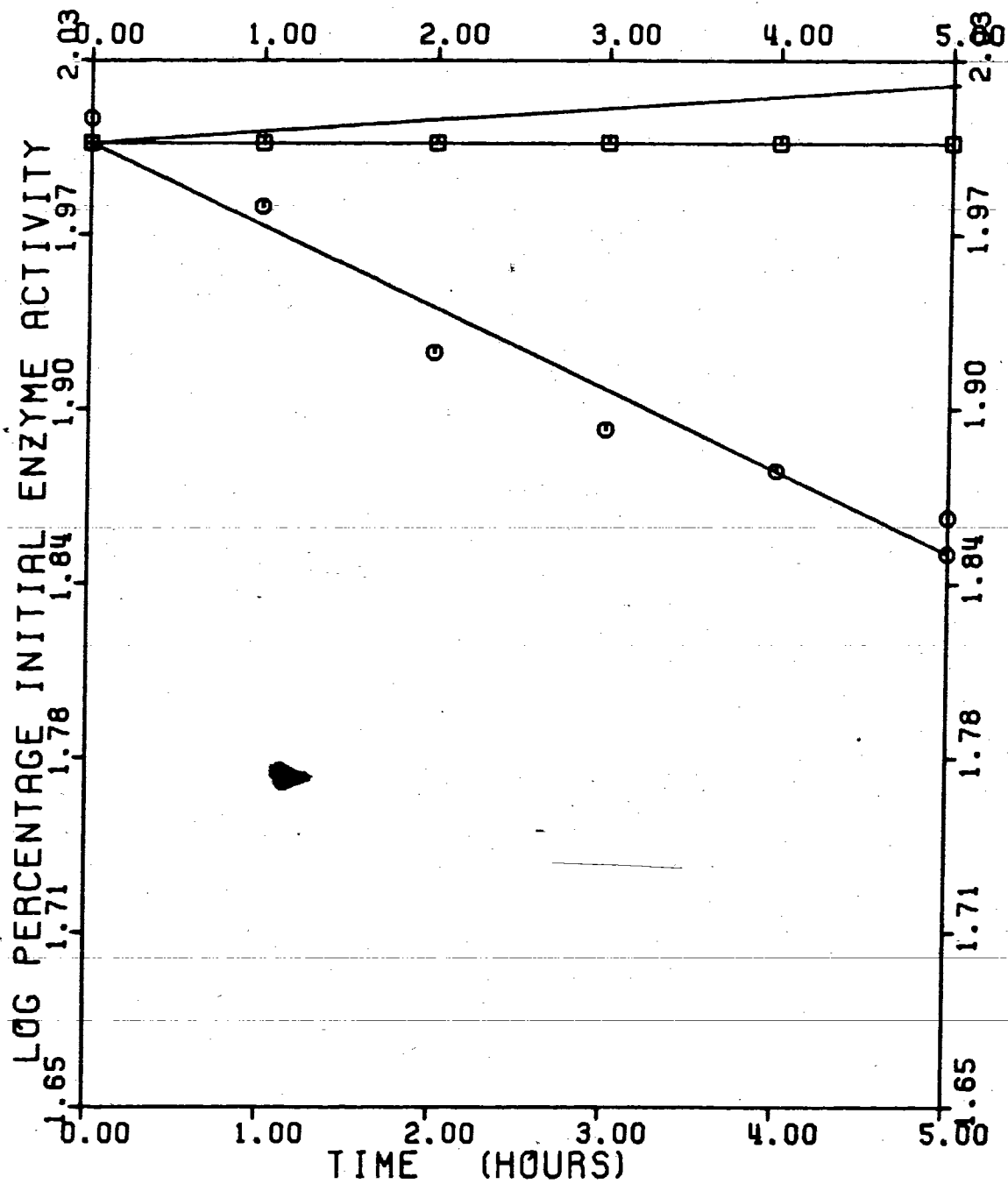
BIBLIOGRAPHY

Adams, G.E., Wilson, R.L., Aldrich, J.E., Cundall, R.B.,
1969, Int.J.Radiat.Biol., 16:333-342. On the mechanism of
radiation-induced inactivation of lysozyme in dilute aqueous
solution.

Adams, G.E., Wilson, R.L., Bisby, R.H., Cundall, R.B., 1971,
Int.J.Radiat.Biol., 20:405-415. On the mechanism cf the
radiation-induced inactivation of ribonuclease in dilute
aqueous solution.

Adams, G.E., Redpath, J.L., Bisby, R.H., Cundall, R.B.,
1972, Israel J.Chem., 10:1079-1093. The use cf free radical
probes in the study cf mechanisms of enzyme inactivaticn.

Adams, G.E., Baverstock, K.F., Cundall, R.B., Redpath, J.L.,
1973, Radiat.Res., 54:375-387. Radiation effects on
alpha-chymotrypsin in aqueous solution: pulse radiolysis
and inactivation studies.

Alder, H.J., 1963, Radiat.Res.Suppl.3, p.110-129. Catalase,
hydrogen peroxide and ionising radiaticn.

Allen, A.O., 1961, The Radiation Chemistry cf Water and
Aqueous Solutions. p.155. D.Van Nostrand Comp., Inc.

Anbar, M., Neta, P., 1967, Int.J.Appl.Radiat.Isotopes,
18:493-523. A compilation of specific bimolecular rate
constants for the reactions of hydrated electrons, hydrogen
atcms and hydroxyl radicals with inorganic and organic
compounds in aquecus solutions.

Bannister, J., Bannister, W., Wood, E., 1971, Eur.J.Biochm.
18:178-186. Bovine erythrocyte cupro-zinc protein. 1.
Isolation and general characterization.

Barber, D.J.W., Thomas, J.K., 1978, Radiat.Res., 74:51-65.
Reactions of radicals with lecithin bilayers.

Behar, D., Czapski, G., Rabani, J., Dorfman, L.M., Schwarz, H.A., 1970, J.Phys.Chem., 74:3209-3213. The acid dissociation constant and decay kinetics of the perhydroxyl radical.

Bielski, B.H.J., Richter, H.W., 1977, J.Am.Chem.Soc., 99:3019-3023. A study of O2- radical chemistry by stopped-flow radiolysis and radiation induced oxygen consumption.

Bisby, R.H., Cundall, R.B., Wardman, P., 1975, Biochim.Biophys.Acta., 389:137-144. A pulse radiolysis study of some free radical reactions with erythrocyte membranes.

Bland, J., Canfield, W., Kennedy, T., Vincent, J., Wells, R., 1978, Physiol.Chem. and Physics, 10:145-152. Effect of tocopherol on photooxidation rate of human erythrocyte membrane IN VITRO.

Bramley, T.A., Coleman, R., Finean, J.B., 1971, Biochim.Biophys.Acta, 241:752-769. Chemical, enzymological and permeability properties of human erythrocyte ghosts prepared by hypotonic lysis in media of different osmolarities.

Bramley, T.A., Coleman, R., 1972, Biochim.Biophys.Acta, 290:219-228. Effects of inclusion of Ca2+, Mg2+, EDTA or EGTA during the preparation of erythrocyte ghosts by hypotonic haemolysis.

Bray, R.C., 1970, Biochem.J., 117:13p. Free-radical intermediates in the reduction of oxygen.

Buchanan, J.D., Armstrong, D.A., 1978, Int.J.Radiat.Biol., 33:410-419. The radiolysis of glyceraldehyde-3-phosphate dehydrogenase.

Buettner, G.R., Oberley, L.W., Chan-Leuthauser, S.W.H., 1978, Photochem.Photobiol., 28:693-695. The effect of iron on the distribution of superoxide and .OH radicals as seen by spin-trapping and on the superoxide dismutase assay.

Carter, J.R.jr., 1973, Biochm., 12:171-176. Role of
sulphydryl in membrane structure.

Conway, A., Koshland, D.E., 1968, Biochem. N.Y.,
7:4011-4023. Negative cooperativity in enzyme action. The
binding of diphosphopyridine nucleotide to
glyceraldehyde-3-phosphate dehydrogenase.

Davison, A., Popma, A., 1978, Int.Congr.Nutrition, 10:146.
Effects of excess retinol, tocopherol, or ascorbate on lipid
autoxidation, enzyme inactivation and permeability of the
plasma membrane of erythrocyte ghosts.

Davison,A.J., Kaminsky, L.S., 1974, Proc.5th.Int.Hyperbaric
Congress (Vancouver), Trapp, W., Bannister, E., Davison, A.,
Trapp, P. (eds.), Simon Fraser Press. Involvement of
oxygen radicals in radiation damage.

Dodge, J.T., Mitchell, C., Hanahan, D.J., 1963,
Arch.Bioch.Biophys., 100:119-130. The preparation and
chemical characteristics of hemoglobin-free ghosts of human
erythrocytes.

Draganic, I.G., Draganic, Z.D., 1969a, J.Phys.Chem.,
73:2571-2577. On the origin of primary hydrogen peroxide
yield in the gamma radiolysis of water.

Draganic, I.G., Nenadovic, M.T., Draganic, Z.D., 1969b,
J.Phys.Chem., 73:2564-2571. Radiolysis of HCOOH + O2 at pH
1.3-13 and the yields of primary products in gamma
radiolysis of water.

Draganic, I.G., Draganic, Z.D., 1971, The Radiation
Chemistry of Water. Academic Press.

Draganic, Z.D., Draganic, I.G., 1971, J.Phys.Chem.,
75:3950-3957. Studies of formation of primary yields of
hydrogen peroxide and molecular hydrogen in the gamma
radiolysis of neutral aqueous solutions.

Draganic, Z.D., Draganic, I.G., 1972, J.Phys.Chem., 1972,
76:2733-2737. Studies on the formation of primary hydrogen
atom yield in the gamma radiolysis of water.

Draganic, Z.D., Draganic, I.G., 1973, J.Phys.Chem.,
77:765-772. Studies on the formation of primary yields of
hydroxyl radical and hydrated electron in the gamma
radiolysis of water.

Duchon, G., Collier, B., 1971, J.Membr.Biol., 6:138-157.
Enzyme activities of human erythrocyte ghosts : effects of
various treatments.

Faraggi, M., Bettleheim, A., 1977, Radiat.Res., 72:81-88.
The reaction of the hydrated electron with amino acids,
peptides, and proteins in aqueous solutions.

Flossmann, W., Westhof, E., 1978, Radiat.Res., 73:75-85.
The action of ionising radiation on protein. II. Radical
formation in L-tryptophan-HCl.

Friedman, M., 1973, The chemistry and biochemistry of the
sulphydryl group in amino acids, peptides and proteins.
(Pergamon)

Frimer,A.A., Rosenthal, L., 1978, Photochem.Photobiol.,
28:711-719. chemical reactions of superoxide anion radical
in aprotic solvents.

George, P., 1965, Oxidases and related redox systems, 1:3,
ed. by King, T.E., Mason, H.S., and Morrison, M., N.Y.,
John Wiley and Sons Inc.

Goldstein, B.D., 1974, Brit.J.Haematol., 27:533. Effects of
irradiation on red cells.

Herbert, D., Pinsent, J., 1948, Biochem.J., 43:203-205.
Crystalline human erythrocyte catalase.

Kaschnitz, R.M., Hatefi, Y., 1975, Arch.Biochem.Biophys.,
171:292-304. Lipid oxidation in biological membranes.

Kellogg III, E.W., Fridovich, I., 1975, J.Biol.Chem., 250:8812-8817. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system.

Kellogg III, E.W., Fridovich, I., 1977, J.Biol.Chem., 252:6721-6728. Liposome oxidation and erythrocyte lysis by enzymatically generated superoxide and hydrogen peroxide.

Klebanoff, S.J., 1958, J.Gen.Physiol., 41:725-753. The effects of x-radiation on the glutathione metabolism of intact erythrocytes IN VITRO.

Kong, S.K., M.sc. thesis, 1979, Simon Fraser University, British Columbia, Canada. The effects of free radicals and oxygen on the permeability and activity of glyceraldehyde-3-phosphate dehydrogenase of resealed erythrocyte ghosts.

Koppenol, W.H., Butler, J., 1977, FEBS Letter, 83:1-6. The reactions involving singlet oxygen and superoxide anion.

Koter, M., Leyko, W., 1973, Annals Med.Sect.Pol.Acadi.Sci., 18:56-58. The effect of gamma-irradiation on ultrastructure of human erythrocyte ghosts.

Lamola, A.A., Yamane, T., Trozzolo, A.M., 1973, Science, 179:1131-1133. Cholesterol hydroperoxide formation in red cell membranes and photohemolysis in erythropoietic protoporphyria.

Lichtin, N.N., Ogdan, J., Stein, G., 1972, Biochim.Biophys.Acta., 276:124-142. Fast consecutive radical processes within the ribonuclease molecule in aqueous solution. II. Reaction with .OH radicals and hydrated electron.

Lin, W.S., Armstrong, D.A., 1978, Int.J.Radiat.Biol., 33:231-243. Effects of superoxide dismutase, dithiothreitol and formate ion on the inactivation of papain by hydroxyl and superoxide radicals in air-saturated solutions.

Lynch, R.E., Fridovich, I., 1978a, J.Biol.Chem., 253:4697-4699. Permeation of the erythrocyte stroma by superoxide radical.

Lynch, R.E., Fridovich, I., 1978b, J.Biol.Chem., 253:1838-1845. Effects of syperoxide radical on the erythrccyte membrane.

Mason, H.S., 1965, A.Rev.Biochem., 34:595-634. Cxidases.

Masuda, T., Ovadia, J., Grossweiner, L.I., 1971, Int.J.Radiat.Biol., 20:447-459. The pulse radiolysis and inactivation cf trypsir.

McCord, J.M., Fridovich. I., 1969, J.Biol.Chem., 244:6049-6055. Superoxide dismutase, an enzymatic function for erythrccuprein.

Mittal, J.P., Hayon, E., 1974, J.Phys.Chem., 78:1790-1794. Interaction of hydrated electrons with phenylalanine and related compounds.

Neta, P., Simic, M., Hayon, E., 1970, J.Phys.Chem., 74:1214-1220. Pulse radiolysis of aliphatic acids in aqueous solution. III. simple amino acids.

Petkau, A., Chelack, W.S., 1976, Biochim.Biophys.Acta, 433:445-456. Radioprotective effect of superoxide dismutase on model phospholipid membranes.

Popma, M.A., M.sc. thesis, 1974, Simon Fraser University, British Columbia, Canada. The effects of retinol, alpha-tocopherol and ascorbic acid on the plasma membrane of erythrocyte ghosts.

Rabani, J., Klug, D., Fridovich, I., 1972, Israel J.Chem., 10:1095-1106. Decay of the HO2 and O2- radicals catalysed by superoxide dismutase. A pulse radiolytic investigation.

Robinson, J.D., 1965, Arch.Bioch.Biophys., 112:170-179.
Structural changes in microsomal suspensions. III.
Formation of lipid peroxides.

Robinson, J.D., 1966, Nature (London), 212:199-120.
Interaction between sulphydryl groups and lipid double bonds
in biological membranes.

Rustgi, S., Joshi, A., Riesz, P., 1977, Int.J.Radiat.Biol.,
32:533-552. E.s.r. of spin-trapped radicals in aqueous
solutions of amino acids. Reaction of the hydrated
electron.

Sokal, R.R., Rohlf, F.J., 1969, Biometry, the principles and
practice of statistics in biological research, Freeman and
Co., p.448-458.

Steck, T.L., Kant, J.A., 1974, Methods Enzymol., 31:172-180.
Preparation of impermeable ghosts and inside-out vesicles
from human erythrocyte membranes.

Sutton, H.C., Roberts, P.B., Winterbourn, C.C., 1976,
Biochem.J., 155:503-510. The rate of reaction of superoxide
radical ion with oxyhaemoglobin and methaemoglobin.

Svingen, B.A., O'Neal, F.O., and Aust, S.D., 1978,
Photochem.Photobiol., 28:803-809. The role of superoxide
and singlet oxygen in lipid peroxidation.

Swallow, A.J., Radiation Chemistry, 1973, John Wiley and
Sons, N.Y.

Tappel, A.L., Adhikari, H.R., 1975, Radiat.Res., 61:177-183.
Fluorescent products from irradiated amino acids and
proteins.

Teipel, J., Koshland, D.E., 1970, Biochim.Biophys.Acta.,
198:183-191. The effect of NAD+ on the catalytic efficiency
of glyceraldehyde-3-phosphate dehydrogenase from rabbit
muscle.

Yonei, S., Todo, T., and Kato, M., 1979, Int.J.Radiat.Biol., 35:161-170. Radiation effects on erythrocyte membrane structure studied by the intrinsic fluorescence .

Whittam, R., 1964, Transport and Diffusion in Red Blood Cells. Edward Arnold Ltd., London, p.76-96.

Zipursky, A., Stephens, M., Brown, E.J., Larsen, P., 1974, J.Clin.Invest., 53:805-812. Sulphydryl groups of the erythrocyte membrane and their relation to glycolysis and drug-induced hemolytic anaemia.

SECTION 4

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 occurence 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 acccunted 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 than
that predicted on the basis of independent actions of
constituent free radicals: (1) H2O2 + O2-, and (2)
oxidizing radicals (.OH, H2O2) + reducing radicals (e-, H.).
These results suggest a Haber-Weiss like interaction between
H2O2 and O2- and an interaction 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
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 O_2-. 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 H_2O_2 and O_2- 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

1971). 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:

$$.OH + H_2O_2 \longrightarrow H_2O + HO_2. \qquad (1)$$

$$HO_2. + H_2O_2 \longrightarrow .OH + H_2O + O_2 \qquad (2)$$
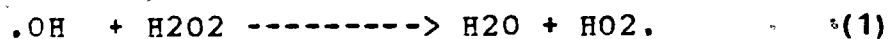
were proposed originally by Haber and Willstatter in 1931 as chain reactions in a mechanism for the decomposition of H_2O_2 initiated by iron ions. These reactions became later known as the "Haber-Weiss cycle" (Haber and Weiss 1934). Reaction (2) has frequently been invoked (Beauchamp and Fridovich

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 O2- or H2O2 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, Foos, Houee and Pucheault 1978,
Koppenol, Butler and van Leeuwen 1978, Dainton and Rowbottom
1953, Czapski and Allen 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, O2- reduces an
iron (III) (or other transition metal) chelate. The reduced
metal may then react with H2O2 to produce .OH, thus:

$$M(N)+ \ + \ O2- \ ---------> \ M(N-1)+ \ + \ O2 \qquad (3)$$

$$M(N-1)+ \ + \ H2O2 \ ---------> \ M(N)+ \ + \ .OH \ + \ OH- \qquad (4)$$
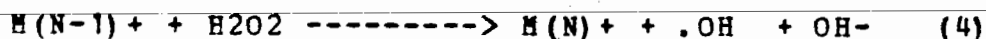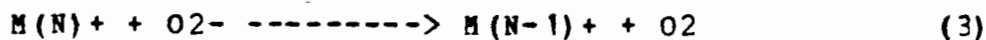
In view of the abundance of metalloproteins and complexed metals in biological tissues, reactions (3) and (4) may well occur in processes causing damage to biological systems.

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 occurence 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 ug/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 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 these 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(mb) and R(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) H_2O_2 + .OH
+ O_2-, (2) .OH + H_2O_2. and interactions decreasing damage
were noted in (3) H_2O_2 + O_2- in the presence of formate, (4)
oxidizing radicals (.OH + H_2O_2) + reducing radicals radicals
($e-$ + H.).

3.1. Mixtures of .OH, H_2O_2 and O_2-.

The observed rates of damage to the permeability of
membranes and to membrane-bound G3PDH by H_2O_2 and O_2- 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 .OH, H_2O_2 and 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 krads of irradiation. As pointed out in section 2, anaerobic media contained 25 uM 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 H202 and .OH was found tc 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 H202 is not likely. Therefcre the enhanced rate would
not be due to direct interaction between .OH and H202.
Powers (1972) has observed similar synergistic effects of
H202 and .OH in his study of radiation damage to cells. He
suggested that organic radicals were produced in the ccurse
of abstraction of H by .OH in reaction (5), with .OH then
being regenerated by reaction (6).

RH2 + .OH --------> RH. + H2O   (5)

RH. + H202 ---------> R + .OH + OH- + H+   (6)

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 is 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% (Kong 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 cf 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 conventicnal 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, O_2- and H_2O_2 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 O_2- and H_2O_2. 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 H_2O_2 in causing damage to the membrane and the known effectiveness of O_2- in inactivating G3PDH (Kong 1979). The present results thus support the occurence 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 O_2- or H_2O_2 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

Rates 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 rereduction 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 7.9-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_2O_2) and reducing
radicals to one of predominantly oxidizing radicals (.OH,
H_2O_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_2O_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 O2-. 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 quantitive 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 O_2- and H_2O_2. 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 N_2 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 H_2O_2 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 $R(enz)$ 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 cf 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 irradiaticn. Conditions were: 0.01 M.
phosphate buffer pH 7.4 rendered isotonic by the addition of
NaCl to 300 mosM., temperature 25 deg C, concentraticn 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 sclutions. 'Organic radicals' means
those formed in the membranes due to direct interaction of
membrane ccmponents 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 1. EFFECTS OF SCAVENGERS ON THE YIELDS OF INDIVIDUAL
FREE RADICALS AND ON THE RATES OF INCREASE IN PERMEABILITY.

scavengers present during irradiation	yields of free radicals (G values)					slope x 10*-3 hcur-1
	.OH	H2O2	O2-	e-	CO2-	
formate		0.6	6.2			1.70+/-0.53
formate, dismutase		3.7				3.50+/-1.6
formate, catalase			6.2			3.93+/-1.07
02, catalase dismutase, formate	(organic radicals)					4.17+/-1.24
catalase, dismutase	2.8					5.05+/-1.41 (initial) 15.92+/-5.07 (final)
N2, formate		0.6		2.8	3.4	5.45+/-1.33
N2	2.8	0.6		2.8	(H)0.6	5.79+/-0.67
catalase	2.8		3.4			6.63+/-0.72
dismutase	2.8	2.3				11.26+/-1.76
no scavengers	2.8	0.6	3.4			16.45+/-3.15
02	2.8	0.6	3.4			2.75+/-3.29 (initial) 986+/-233 (final)
02, formate		0.6	6.2			1.95+/-1.38 (initial) 13.5+/-5.1 (final)
02, catalase,	2.8					47.50+/-9.01 (initial) 1577+/-8.75 (final)

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 2. EFFECTS OF SCAVENGERS ON RATES OF INACTIVATION OF MEMBRANE-BOUND G3PDH.

scavengers present during irradiation	yields of free radicals in G values					slope x 10*-3 hour-1
	.OH	H2O2	O2-	e-	CO2-	
N2,formate		0.6		2.8	3.4	6.40+/-2.27
formate		0.6	6.2			19.24+/-4.72
formate, dismutase		3.7				29.74+/-3.90
catalase, dismutase	2.8					32.47+/-3.68
O2, catalase, dismutase, formate	(organic radicals)					35.47+/-6.20
dismutase	2.8	2.3				43.97+/-3.77
catalase	2.8		3.4			47.27+/-5.96
formate, catalase			6.2			53.93+/-8.76
N2	2.8	0.6		2.8 (H)	0.6	55.50+/-7.62(initial) 0 (final)
no scavengers	2.8	0.6	3.4			84.28+/-7.99
O2	2.8	0.6	3.4			119.5+/-16.5(initial) 229.8+/-11.6(final)
O2, formate		0.6	3.4			31.13+/-8.86(initial) 81.33+/-13.88(final)
O2,catalase, dismutase	2.8					178.93+/-25.83

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 3. SUMMARY OF EFFECTS OF INTERACTIONS BETWEEN FREE
RADICALS CN THE RATES OF INCREASE OF PERMEABILITY.

scavengers present during irradiation	rate of increase in permeability $\times 10^{-3}$ hour^{-1}		
	experimental	calculated	exp/calc
formate	1.70+/-0.53	4.48+/-1.67	*0.38+/-0.26
N2	5.79+/-0.67	8.60+/-3.07	0.67+/-0.32
catalase	6.63+/-0.72	7.18+/-2.56	0.92+/-0.43
dismutase	11.26+/-1.76	7.23+/-2.97	*1.56+/-0.89
no scavengers	16.45+/-3.15	7.75+/-2.86	*2.12+/-1.19

TABLE 4. EFFECTS CF INTERACTIONS BETWEEN FREE RADICALS ON THE RATES OF DAMAGE TC 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 cf 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 cf 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.

scavengers present irradiation	rate of inactivation of G3PDH during x 10*-3 hour-1		
	experimental	calculated	exp/calc
formate	19.24+/-4.12	58.70+/-13.94	*0.33+/-0.16
N2	0	39.68+/-8.23	*0
catalase	47.27+/-5.96	62.03+/-13.26	0.76+/-0.25
dismutase	43.97+/-3.77	50.97+/-10.05	0.86+/-0.25
no scavengers	84.28+/-7.99	66.85+/-14.27	*1.26+/-0.38

TABLE 5. THE EFFECTS OF OXYGEN ON THE RATES 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 RATES OF INCREASE IN PERMEABILITY OF GHOSTS

scavengers present during irradiation	rates of increase of permeability x 10*-3 under the specified atmospheres in irradiation medium			comparison of rates		
	N2	air	O2	air	O2	O2
				N2	N2	air
formate	5.45	1.70	1.95 (initial)	0.3+/-0.2 (initial)	0.4+/-0.4 (initial)	1.2+/-1.2 (initial)
			13.50 (final)		2.5+/-1.6 (final)	7.9+/-5.5 (final)
no scavengers	5.79	16.45	2.75 (initial)	2.8+/-0.9 (initial)	0.47+/-0.5 (initial)	*0.17+/-0.2 (initial)
			986 (final)		170+/-60 (final)	60+/-25 (final)
catalase,	5.0 (initial)	47.50 (initial)				*9.5+/-4.5 (initial)
dismutase	15.92 (final)	1577 (final)				99+/-32 (final)

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 1 in all cases except the ratio with (*).

TABLE 6. THE EFFECTS OF OXYGEN ON THE RATES OF INACTIVATION OF MEMBRANE-BOUND G3PDH.

scavengers present during irradiation	rates of inactivation of G3PDH x 10*-3 under the specified atmospheres in irradiation medium			comparison of rates		
	N2	air	O2	air / N2	O2 / N2	O2 / air
formate	6.40	19.24	34.3 (initial)	3.0+/-1.8	5.4+/-3.3 (initial)	1.8+/-0.9 (initial)
			81.30 (final)		13.0+/-6.8 (final)	4.2+/-1.8 (final)
no scavengers	55.5	84.28	119.5 (initial)	1.5+/-0.3	2.2+/-0.6 (initial)	1.4+/-0.3 (initial)
			229.75 (final)		4.1+/-0.8 (final)	2.7+/-0.4 (final)
catalase, dismutase		32.47	178.9			*5.5+/-1.4

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-BCUND 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-   ⓪

H2O2 + O2-   ▣

.OH + H2O2   ⊠

.OH + O2-   ◈

Organic radicals + O2 ✳

FIGURE 2.   EFFECTS OF MIXTURES OF .OH, H2O2 AND C2- ON THE
ACTIVITY OF MEMBRANE-BOUND G3PDH OF RESEALED ERYTHROCYTE
GHOSTS.
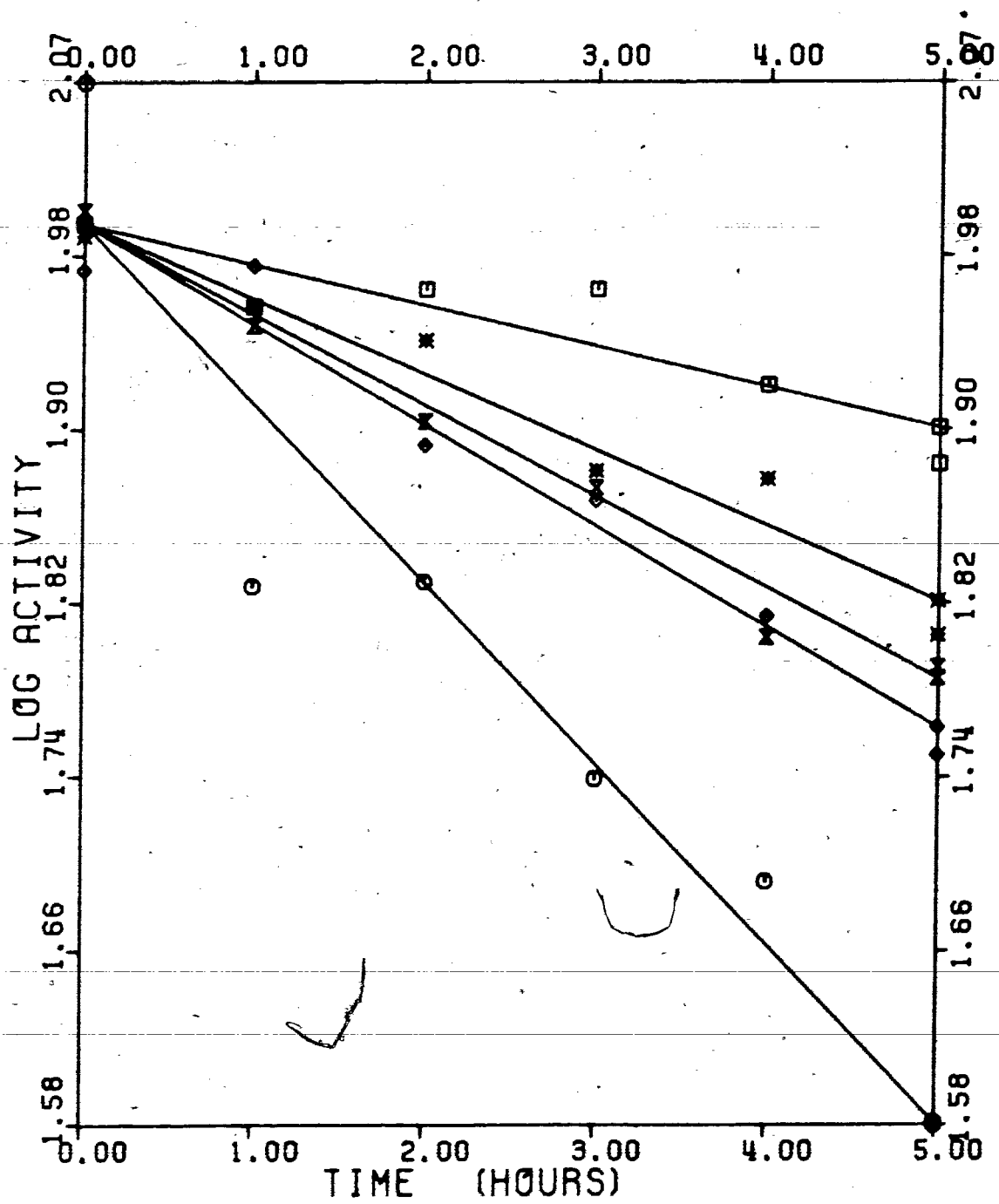
FIGURE 3.   EFFECTS OF MIXTURES OF OXIDIZING FREE RADICALS:
.OH AND H2O2 AND REDUCING FREE RADICALS: e-, .H AND CO2- ON
THE PERMEABILITY OF RESEALED ERYTHROCYTE GHOSTS.   Logarithm
of percentage of ghosts remaining intact after each hour of
irradiation is plotted as a function of time in hour.
conditions were as described in Figure 1.

Symbols

      .OH + H2O2 + e- + .H   ✕

      H2O2 + e- + CO2-   ✳

FIGURE 3.   EFFECTS OF MIXTURES OF OXIDIZING FREE RADICALS:
.CH AND H2O2 AND REDUCING FREE RADICALS: e-, .H AND CO2- ON
THE PERMEABILITY OF RESEALED ERYTHROCYTE GHOSTS.
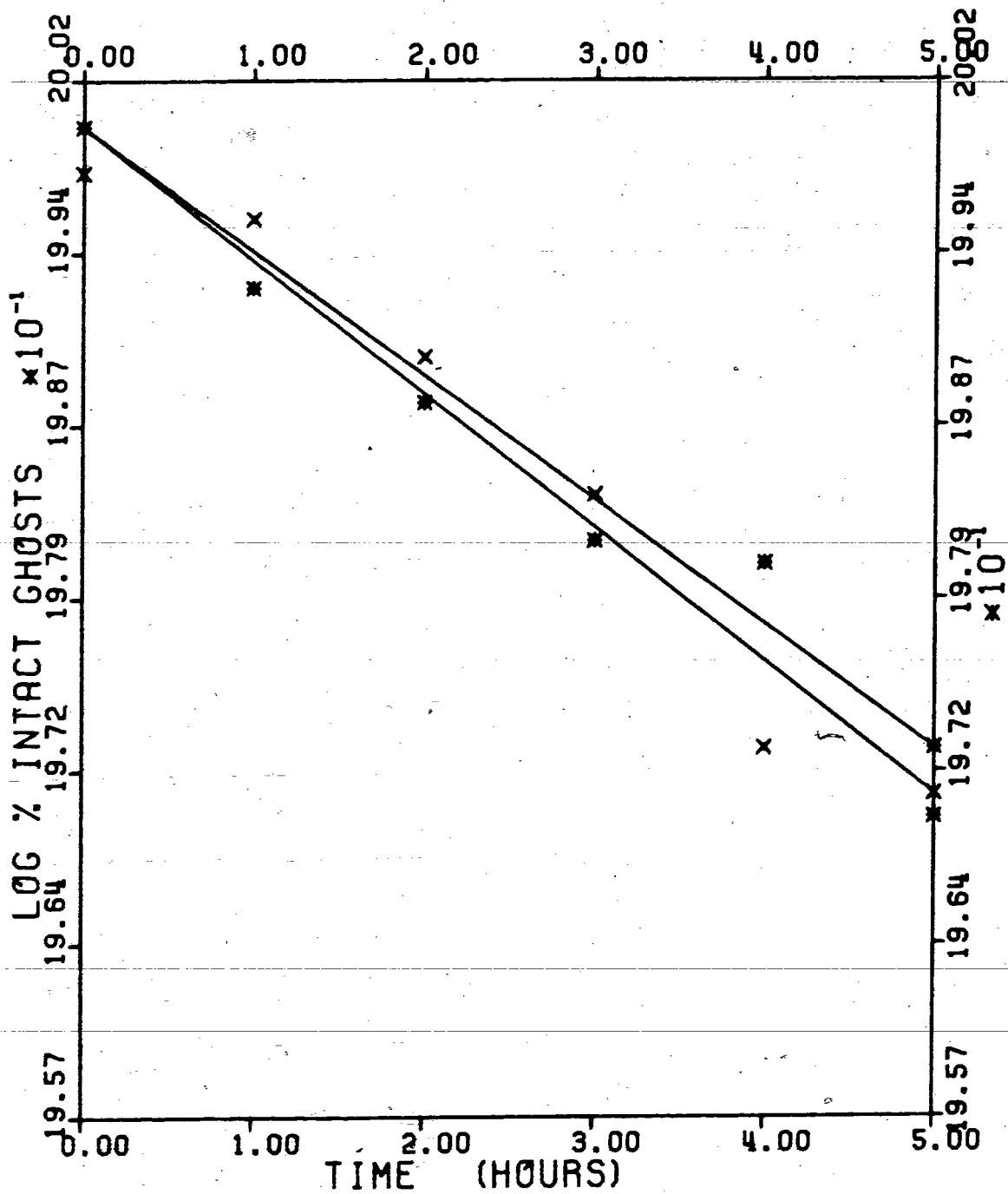
FIGURE 4. EFFECTS OF MIXTURES OF OXIDIZING FREE RADICALS:
.OH AND H2O2 AND REDUCING FREE RADICALS: e-, .H AND CO2- ON
THE ACTIVITY OF MEMBRANE-BOUND G3PDH OF RESEALED ERYTHROCYTE
GHOSTS. Logarithm of percentage of activity of G3PDH
remaining after each hour of irradiation is plotted as a
function of time in hour. conditions were as described in
Figure 1.

Symbols

.OH + H2O2 + e- + .H   X

H2O2 + e- + CO2-   *

FIGURE 4. EFFECTS OF MIXTURES OF OXIDIZING FREE RADICALS:
.OH AND H2O2 AND REDUCING FREE RADICALS: e-, .H AND CO2- ON
THE ACTIVITY OF MEMBRANE-BOUND G3PDH OF RESEALED ERYTHROCYTE
GHOSTS.
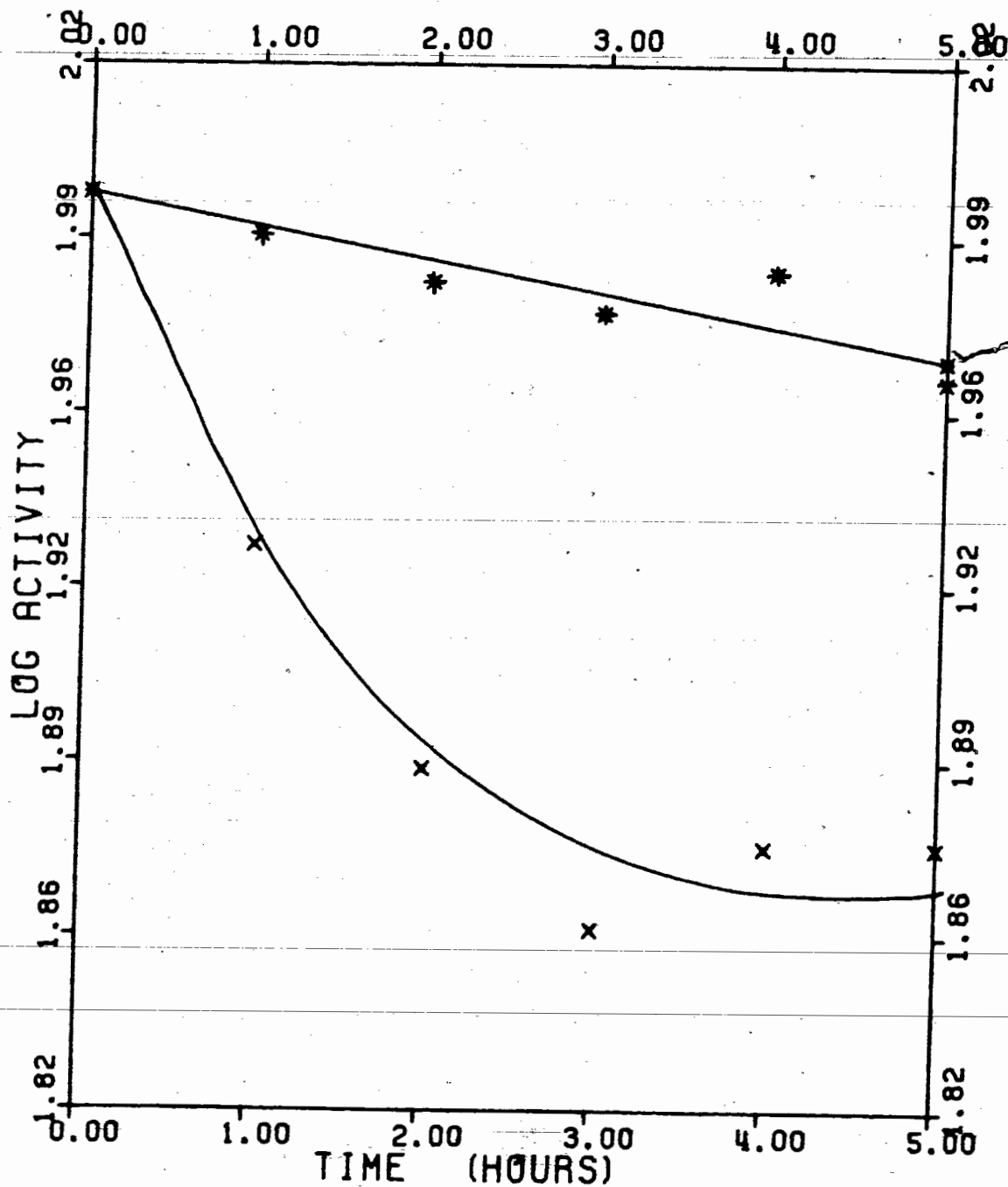
FIGURE 5. EFFECTS OF OXYGEN ON THE DAMAGE BY .OH, H2C2, O2-
AND MIXTURES OF THESE RADICALS ON THE PERMEABILITY OF
RESEALED ERYTHROCYTE GHOSTS. Logarithm of percentage cf
ghosts remaining intact after each hour of irradiation is
plotted as a function cf time in hour. Conditions were as
described in Figure 1.

Symbols

.OH + H2O2 + C2-

.OH + H2O2 + C2- + O2

H2O2 + O2-

H2O2 + O2- + O2

.OH

.OH + O2

FIGURE 5. EFFECTS OF OXYGEN ON THE DAMAGE BY .OH, H2O2, O2-
AND MIXTURES OF THESE RADICALS ON THE PERMEABILITY OF
RESEALED ERYTHROCYTE GHOSTS.
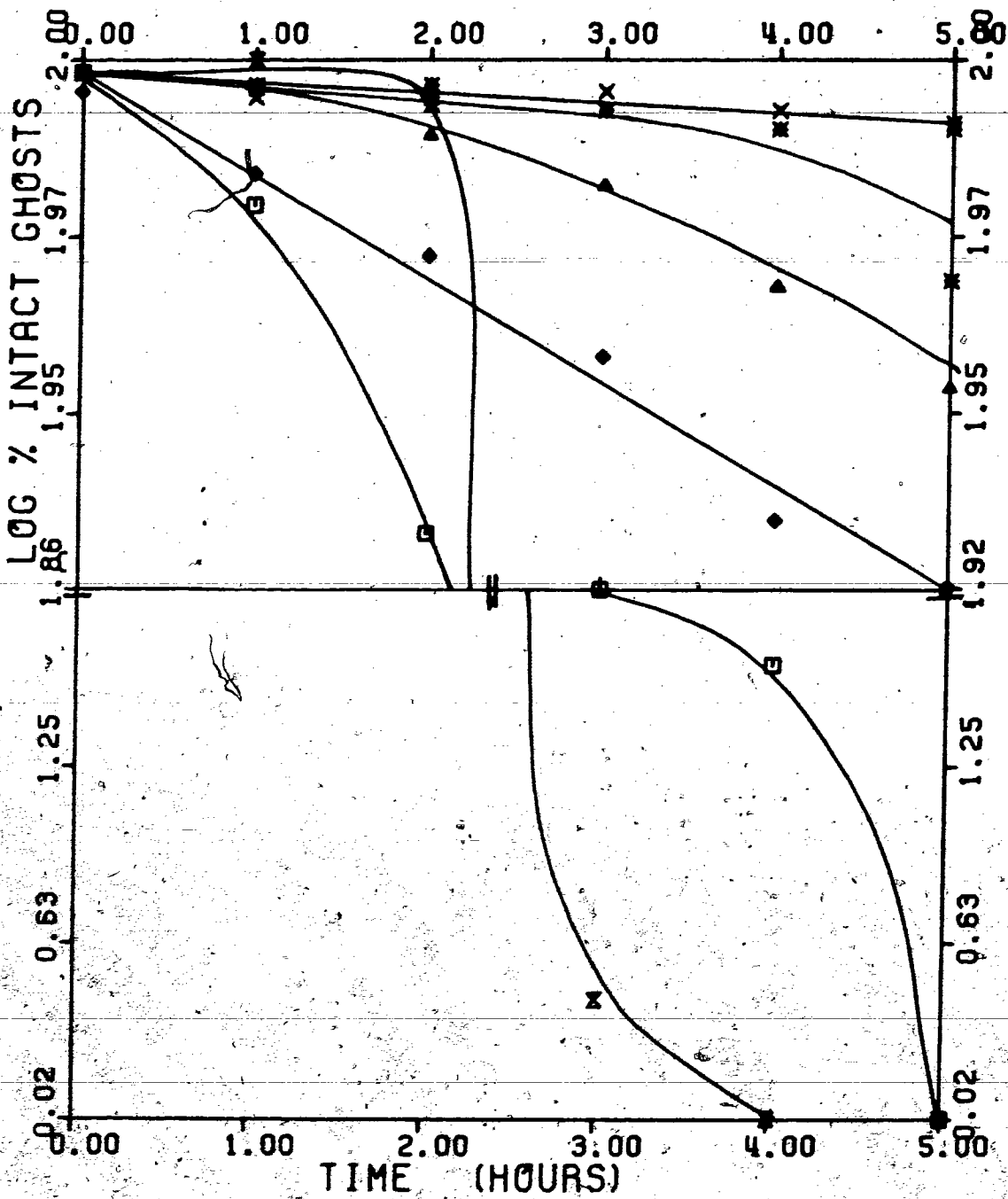
FIGURE 6. EFFECTS OF OXYGEN ON THE DAMAGE BY .OH, H2O2, O2-

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 + O2- + 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.
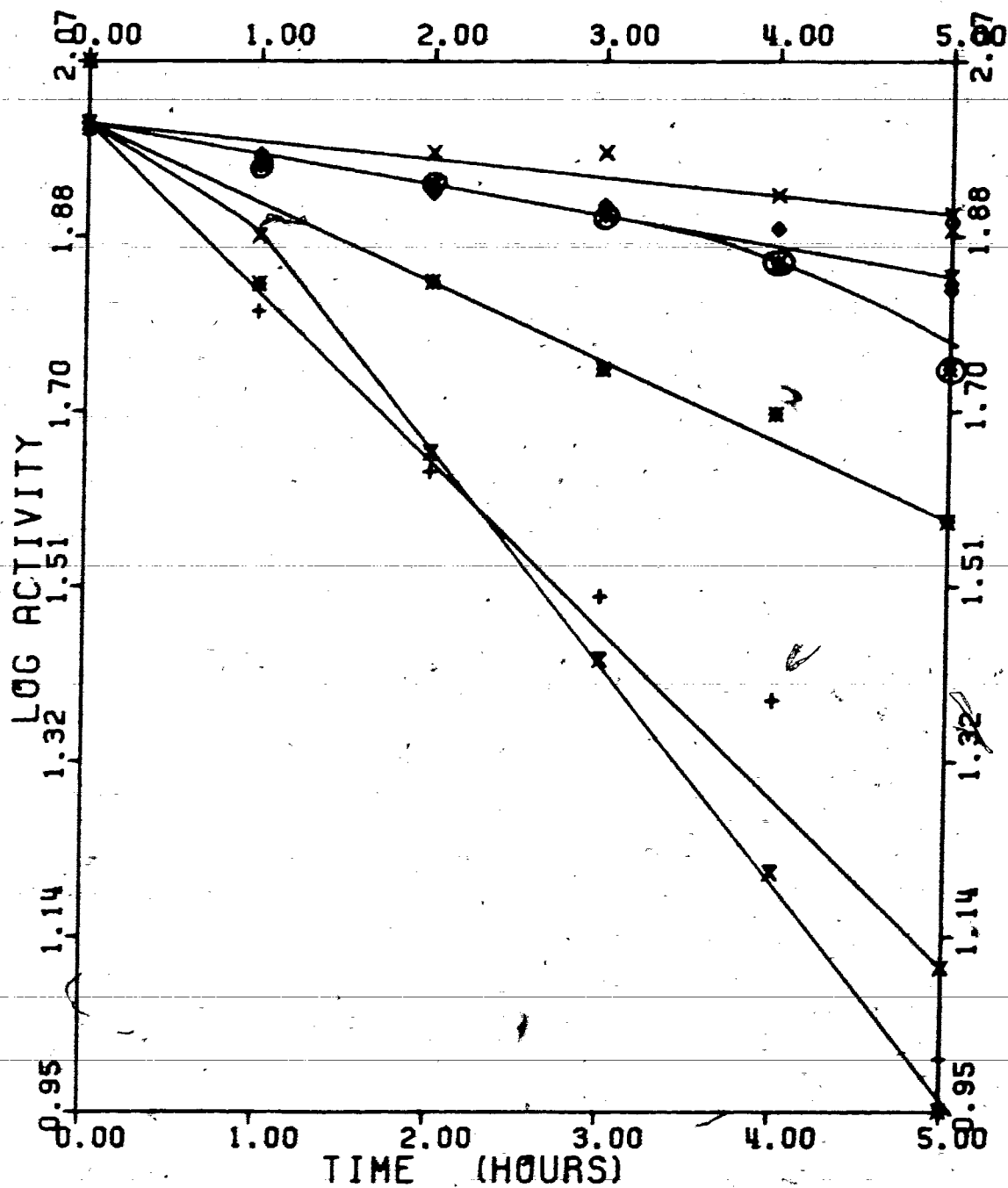
BIBLIOGRAPHY

Adams, G.E., 1972, Adv.Radiat.Chem., 3:125-208. Radiation chemical mechanisms in radiation biology.

Alper, T., 1971, Biophysical Aspects of Radiation Quality, p.171. Vienna.

Alper, T., 1956, Radiat.Res., 5:573-586. The modification of damage caused by primary ionisation of biological targets.

Alper, T., 1968, Nature (London), 217:862-863. Low oxygen enhancement ratios for radiosensitive bacterial strains, and the probable interaction of two types of primary lesion.

Beauchamp, C.O., Fridovich, I., 1970, J.Biol.Chem., 245:4641-4646. A mechanism for the production of ethylene from methional. The generation of .OH radical by xanthine oxidase.

Bramley, T.A., Coleman, R., Finean, J.B., 1971, Biochim.Biophys.Acta, 241:752-769. Chemical, enzymological and permeability properties of erythrocyte ghosts prepared by hypotonic lysis in media of different osmolarities.

Bramley, T.A., Coleman, R., 1972, Biochim.Biophys.Acta, 290:219-228. Effects of inclusion of Ca2+, Mg2+, EDTA or EGTA during the preparation of erythrocyte ghosts by hypotonic haemolysis.

Buchanan, J.D., Armstrong, D.A., 1978, Int.J.Radiat.Biol., 33:410-413. The radiolysis of glyceraldehyde-3-phosphate dehydrogenase.

Cohen, G., Heikkela, R.E., 1974, J.Biochem., 249:2447-2452. The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents.

Czapski, G., Allen, A.C., 1962, J.Phys.Chem., 66:262-266.
Evidence for the superoxide-dependent reduction of Fe3+ and
its role in enzymatic generated .OH radical formation.

Dainton, F.S., Rowbottom, J., 1953, Trans.Farad.Soc.,
49:1160-1173. The primary radical yield in water: a
comparison of photolysis and radiolysis of H2O2.

Davison, A.J., Kaminsky, L.S., 1974, Proc.5th.Int.Hyperbaric
Congress (Vancouver), Trapp, W., Bannister, E., Davison, A.,
Trapp, P. (eds.), Simon Fraser Press. Involvement of
oxygen radicals in radiation damage.

Davison, A., Kong, S.K., 1977, Pacific Slope Biochemical
Conference, p.45. Free radical mechanisms in the effects of
gamma-irradiation on the plasma membranes of resealed
erythrocyte ghosts.

Dodge, J.T., Mitchell, C., Hanahan, D.J., 1963,
Arch.Bioch.Biophys., 100:119-130. The preparation and
chemical characteristics of hemoglobin-free ghosts of human
erythrocytes.

Draganic, I.G., Draganic, Z.D., 1969a, J.Phys.Chem.,
73:2571-2577. On the origin of primary hydrogen peroxide
yield in the gamma radiolysis of water.

Draganic, I.G., Nenadovic, M.T., Draganic, Z.D., 1969b,
J.Phys.Chem., 73:2564-2571. Radiolysis of HCOOH + O2 at pH
1.3-13 and the yields of primary products in gamma
radiolysis of water.

Draganic, Z.D., Draganic, I.G., 1971, J.Phys.Chem.,
75:3950-3957. Studies of formation of primary yields of
hydrogen peroxide and molecular hydrogen in the gamma
radiolysis of neutral aqueous solutions.

Draganic, Z.D., Draganic, I.G., 1973, J.Phys.Chem.,
77:765-772. Studies on the formation of primary yields of
hydroxyl radical and hydrated electron in the gamma
radiolysis of water.

Duchon, G., Collier, B., 1971, J.Membr.Biol., 6:138-157.
Enzyme activities of human erythrocyte ghosts : effects of
various treatments.

Ewing, D., 1978, Radiat.Res., 73:121-136. Additive
sensitization of bacterial spores by oxygen and
p-nitroacetophenone.

Ferradini, C., Foos, J., Houee, C, Pucheault, J., 1978,
Photochem.Photobiol., 28:697-700. The reaction between
superoxide anions and H2O2.

Fong, K.L., McCay, P.B., Poyer, J.L., Misra, H.P., Keele,
B.B., 1976, Chem.Biol.Interact., 15:77-89 Evidence for
superoxide-dependent reduction of Fe3+ and its role in
enzyme-generated .OH radical formation.

Fong, K.L., McCay, P.B., Poyer, J.L., Misra, H.P., Keele,
B.B., 1973, 248:7792-7797. Evidence that peroxidation of
lysosomal membranes is initiated by .OH free radicals
produced during flavin enzyme activity.

Fee, J.A., Teitelbaum, H.D., 1972,
Bioch.Biophys.Res.Commun., 49:150-157. Evidence that
superoxide dismutase plays a role in protecting red blood
cells against peroxidative hemolysis.

Goscin, S.A., Fridovich, I., 1972, Arch.Bioch.Biophys.,
153:778-783. The role of superoxide radical in
non-enzymatic hydroxylation.

Haber,F., Weiss, J., 1934, Proc.R.Soc.London A, 147:332-351.
Catalytic decomposition of hydrogen peroxide by iron salts.

Halliwell, B., 1976, FEBS Lett. 72:8-10. An attempt to
demonstrate a reaction between superoxide and H2O2.

Halliwell, B., 1978, FEBS Lett., 96:238-242.
Superoxide-dependent formation of .OH radicals in the
presence of iron salts. Its role in degradation of
hyaluronic acid by a superoxide generating system.

Hodgson, E.K., Fridovich, I., 1976a, Biochim.Biophys.Acta,
430:182-188. The accumulation of superoxide radical during
aerobic action of xanthine oxidase.

Hodgson, E.K., Fridovich, I., 1976b, Arch.Bioch.Biophys.,
172:202-205. The mechanism of the activity dependent
luminescence of xanthine oxidase.

Howard-Flanders, P., 1960, Nature, 186:485-487. Effect of
oxygen on the radiosensitivity of bacteriophage in the
presence of sulphydryl compounds.

Kellogg III, E.W., Fridovich, I., 1975, J.Biol.Chem.,
250:8812-8817. Superoxide, hydrogen peroxide, and singlet
oxygen in lipid peroxidation by a xanthine oxidase system.

Koch, C.J., Kruuv, J., Frey, H.E., 1973, Radiat.Res.,
53:33-42. Variation in radiation response of mammalian
cells as a function of oxygen tension.

Kong, S.K., M.Sc. thesis, 1979 July, Simon Fraser
University, British Columbia, Canada. The effects of free
radicals and oxygen on the permeability and activity of
glyceraldehyde-3-phosphate dehydrogenase of resealed
erythrocyte ghosts.

Koppenol, W.H., Butler, J., 1977, FEBS Letter, 83:1-6. The
reactions involving singlet oxygen and superoxide anion.

Koppenol, W.H., Butler, J., vanLeenwen, J.W., 1978,
Photochem.Photobiol., 28:655-660. The Haber-Weiss cycle.

McCord, J.M., 1974, Sci., 185:529-531. Free radicals and
inflammation: protection of synovial fluid by superoxide
dismutase.

McCord, J.M., Day, E.D.jr., 1978, FEBS Lett.,86:139-142.
Superoxide-dependent production of .OH radical catalysed by
iron EDTA complexes.

McCord, J.M., Fridovich. I., 1969, J.Biol.Chem.,
244:6049-6055. Superoxide dismutase, an enzymatic function
for erythrocuprein.

McClune, G.J., Fee, J.A., 1976, FEBS Lett., 67:194-298.
Stopped-flow spectrophotometric observation of superoxide
dismutation in aqueous solution.

Michaels, H.B., Hunt, J.W., 1977, Radiat.Res., 72:18-31.
Reaction of oxygen with radiation-induced free radicals on
single-stranded polynucleotides.

Popma, M.A., M.Sc. thesis, 1974, Simon Fraser University,
British Columbia, Canada. The effects of retinol, alpha-
tocopherol and ascorbic acid on the plasma membrane of
erythrocyte ghosts.

Powers, E.L., 1972, Israel J.Chem., 10:1199-1211. The
hydrated electron, the hydroxyl radical and hydrogen
peroxide in radiation damage in cells.

Purdie, J.W., 1971, Radiat.Res., 48:474-483. Investigation
of chain reactions and oxygen effects during radiolysis of
peptide disulphide bonds using cysteine-glutathione
disulphide as a model.

Quitiliani, M., Badiello, R., Tamba, M., Esfandi, A., Gorin,
G., 1977, Int.J.Radiat.Biol., 32:195-202. Radiolysis of
glutathione in oxygen containing solutions of pH 7.

Raleigh, J.A., Kremers, W., Gaboury, B., 1977,
Int.J.Radiat.Biol., 31:203-213. Dose-rate and oxygen
effects in models of lipid membranes : linoleic acid.

Sokal, R.R., Rohlf, F.J., 1969, Biometry, the principles and
practice of statistics in biological research, Freeman and
Co., p.448-458.

Srivastava, B.S., 1974, Int.J.Radiat.Biol., 26:391-394. The yield of single-strand breaks in the DNA of bacteriophage lamda irradiated extra- and intracellularly with gamma-ray in oxic and anoxic conditions.

Steck, T.L., Kant, J.A., 1974, Methods Enzymol., 31:172-180. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes.

Sutton, H.C., Roberts, P.B., Winterbourn, C.C., 1976, Biochem.J., 155:503-510. The rate of reaction of superoxide radical ion with oxyhaemoglobin and methaemoglobin.

Van der Schans, G.P., Blok, J., 1969, Int.J.Radiat.Biol., 17:25-38. The influence of oxygen and sulphydryl compounds on the production of breaks in bacteriophage DNA by gamma-rays.

Van Hemmen, J.J., Meuling, W.J.A., Bleichrodt, J.F., 1978, Radiat.Res. 75:410-423. Effect of oxygen on inactivation of biologically active DNA by gamma rays IN VITRO: influence of metalloporphyrins and enzymatic DNA repair.

Wills, E.D., Wilkinson, A.E., 1967, Radiat.Res., 31:732-747. The effect of irradiation on lipid peroxide formation in subcellular fractions.

Yuhas, J.M., Li, A.P., 1978, Radiat.Res., 75:563-572. In vitro studies on the radioresistance of oxic and hypoxic cells in the presence of both radioprotective and radiosensitizing drugs.

Zimmermann, R., Flohe, L., Weser, U., Hartmann, H.J., 1973, FEBS Letter, 29:117-120. Inhibition of of lipid peroxidation in isolated inner membrane of rat liver mitochondria by superoxide dismutase.

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, O_2- and H_2O_2 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 RESEALED HUMAN ERYTHROCYTE GHOSTS

1.1. Prewashing

150 ml of blood was centrifuged in six 40ml centrifuge
tubes. The serum and buffy coat were aspired 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 spinned 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.

Appendix 2

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 O_2-
generated by aerobic xanthine oxidase system, thus
inhibiting the rate of reduction of cytochrome C by this
system.

Procedure

Into 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:

$$units/mg = \frac{dilution\ of\ SOD\ causing\ a\ rate\ of\ 0.0125/min}{0.1 \times mg\ SOD/\ ml\ original\ solution}$$

Appendix 3

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,

$$\text{unit/mg} = \frac{\text{rate of decrease of absorbance at 240 nm}}{0.043 \times \text{mg enz/ml reaction mixture}}$$

The molar absorption coefficient for H_2O_2 is $0.043 \times 10^{*3}$

Appendix 4
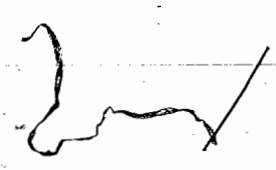
EFFECTS OF TRITON X-100 AND DITHIOTHREITOL ON THE
ACTIVITY OF MEMBRANE-BOUND GLYCERALDEHYDE-3-PHOSPHATE
DEHYDROGENASE.

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 (Tjice 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 per cent
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 acticvity was rapidly restored when concentration of
DTT was increased frcm 50 tc 60 micromolar (Figure 3).
Maximum restoration of activity was reached about 80
micromolar.   No significant increase was observed up tc 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%. DTT 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 cf
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 G3PDH.
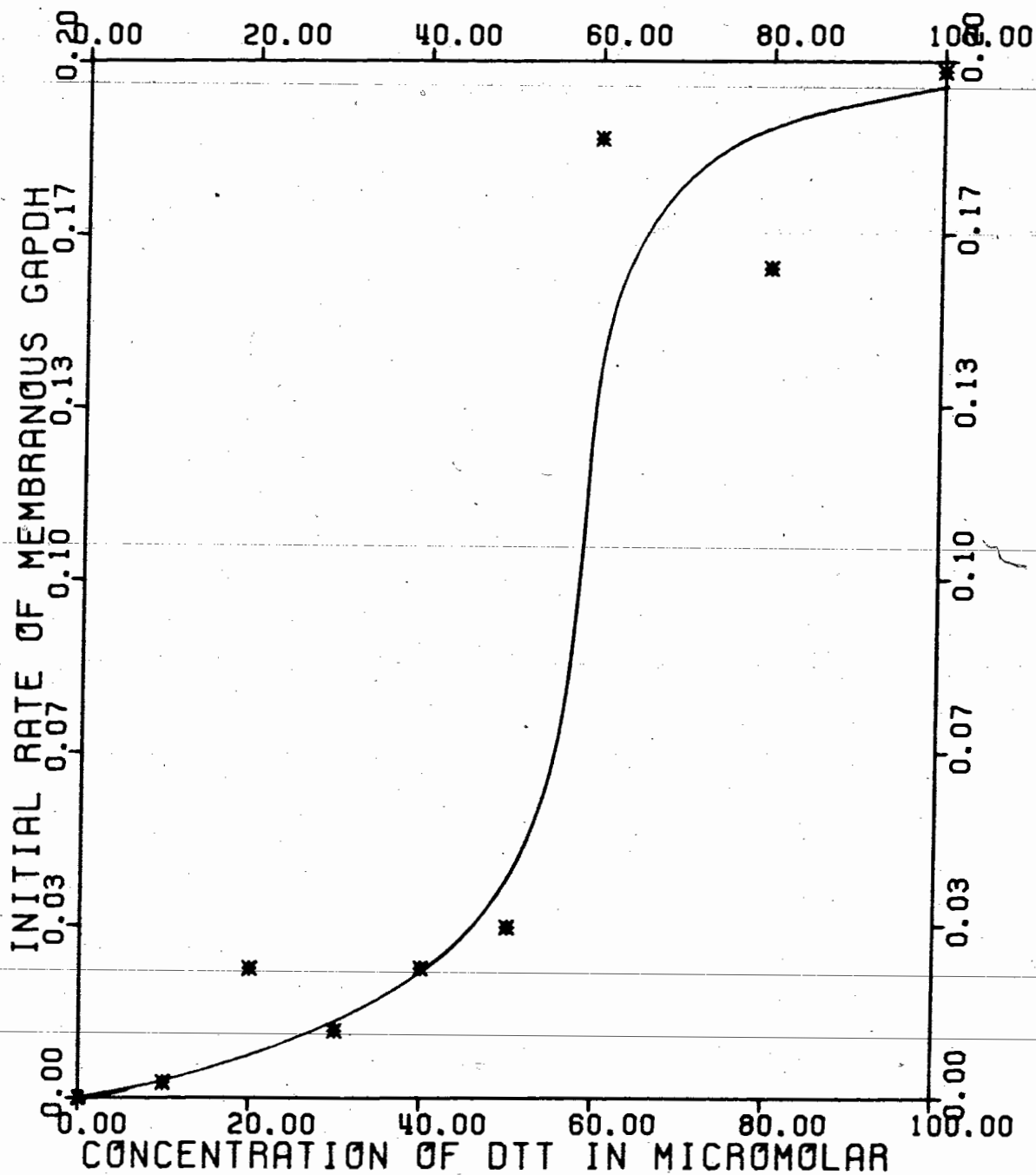
FIGURE 2. EFFECT OF TRITON ON THE RATE OF PRODUCT
INHIBITION ON MEMBRANE-BOUND G3PDH. (Amax-A)/t was plotted
against (Amax-A) for various concentrations of Triton X-100.
Amax is the maximum absorbance at which NAD in the reaction
mixture was exhausted. A is the absorbance at time t.
Conditions were as 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.
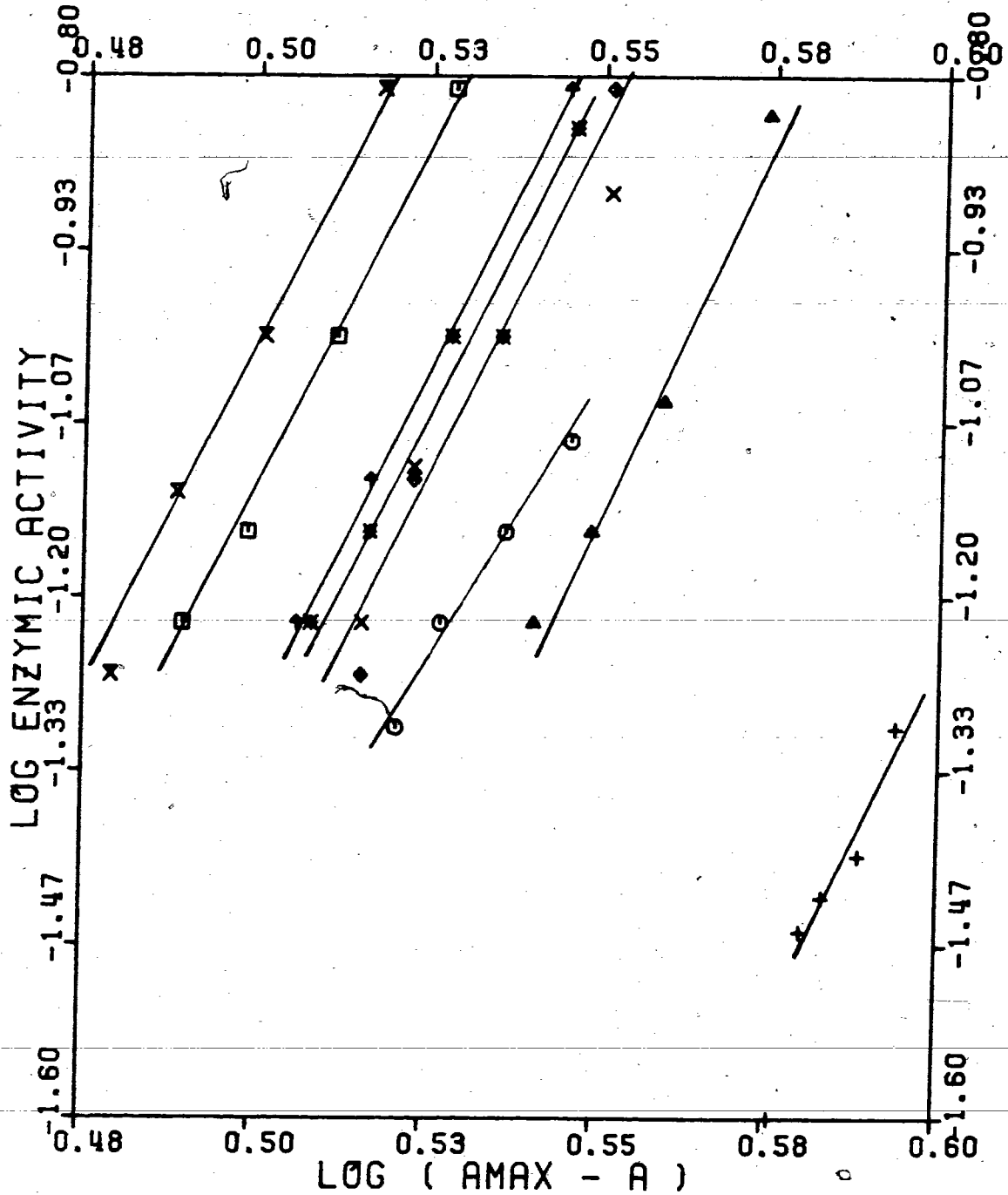
FIGURE 3. EFFECT OF DTT ON INITIAL RATE OF REDUCTION OF NAD
CATALYSED BY MEMBRANE-BOUND G3PDH. Absorbance per minute
was plotted as a function of concentrations of DTT in
micromolar. Conditions were as described in Methods.

FIGURE 3. EFFECT OF DTT ON INITIAL RATE OF REDUCTION OF NAD
CATALYSED BY MEMBRANE-BOUND G3PDH
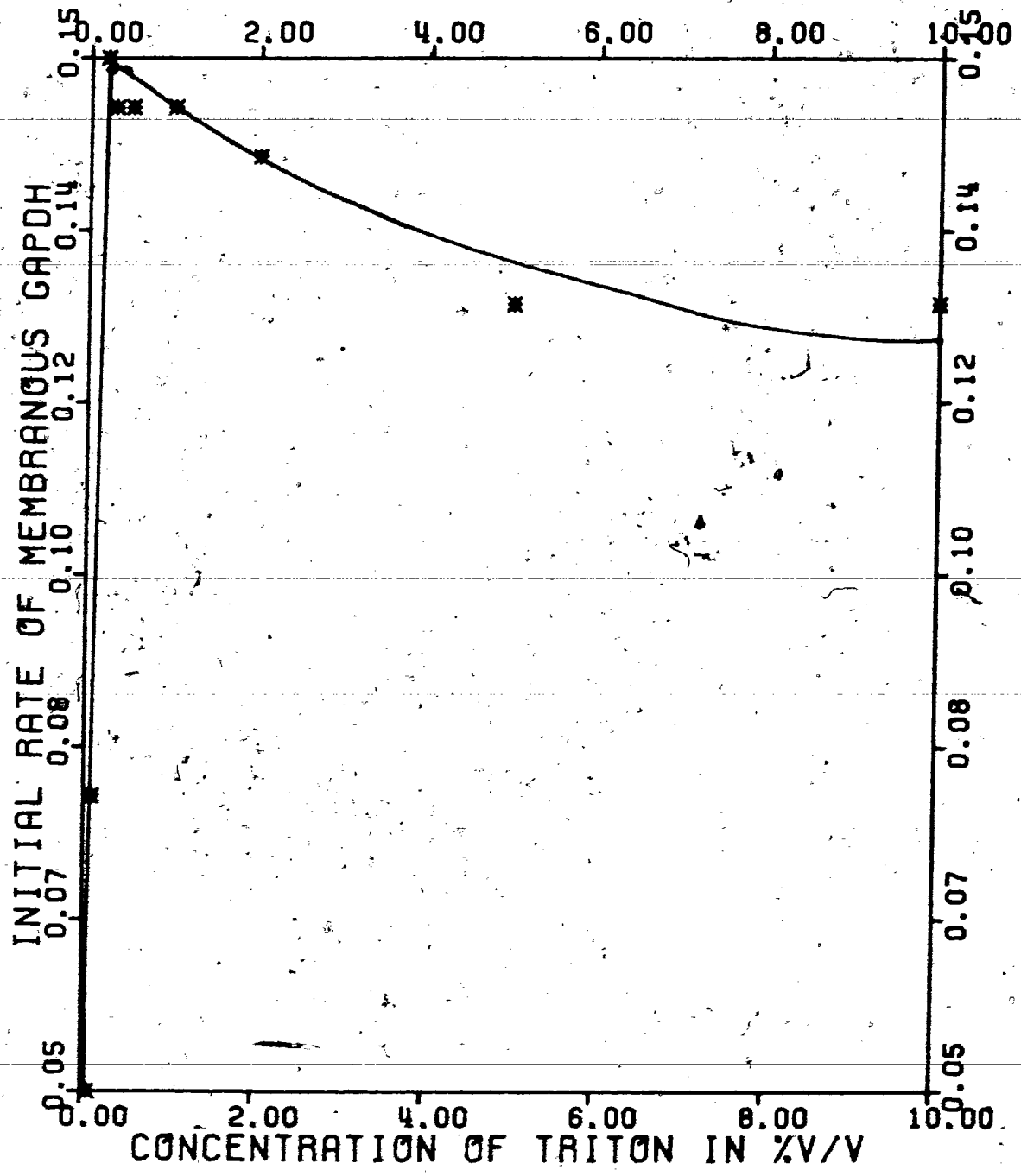
FIGURE 4. EFFECT OF DTT ON THE RATE OF PRODUCT INHIBITION
ON MEMBRANE-BOUND G3PDH. (Amax-A)/t was plotted against
(Amax-A) for various concentrations of DTT. Amax and A were
as defined in Figure 2. Conditions were as described in
Methods. Concentrations of DTT were 20, 30, 40, 50, 60, 80
and 100 micromolar.

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.
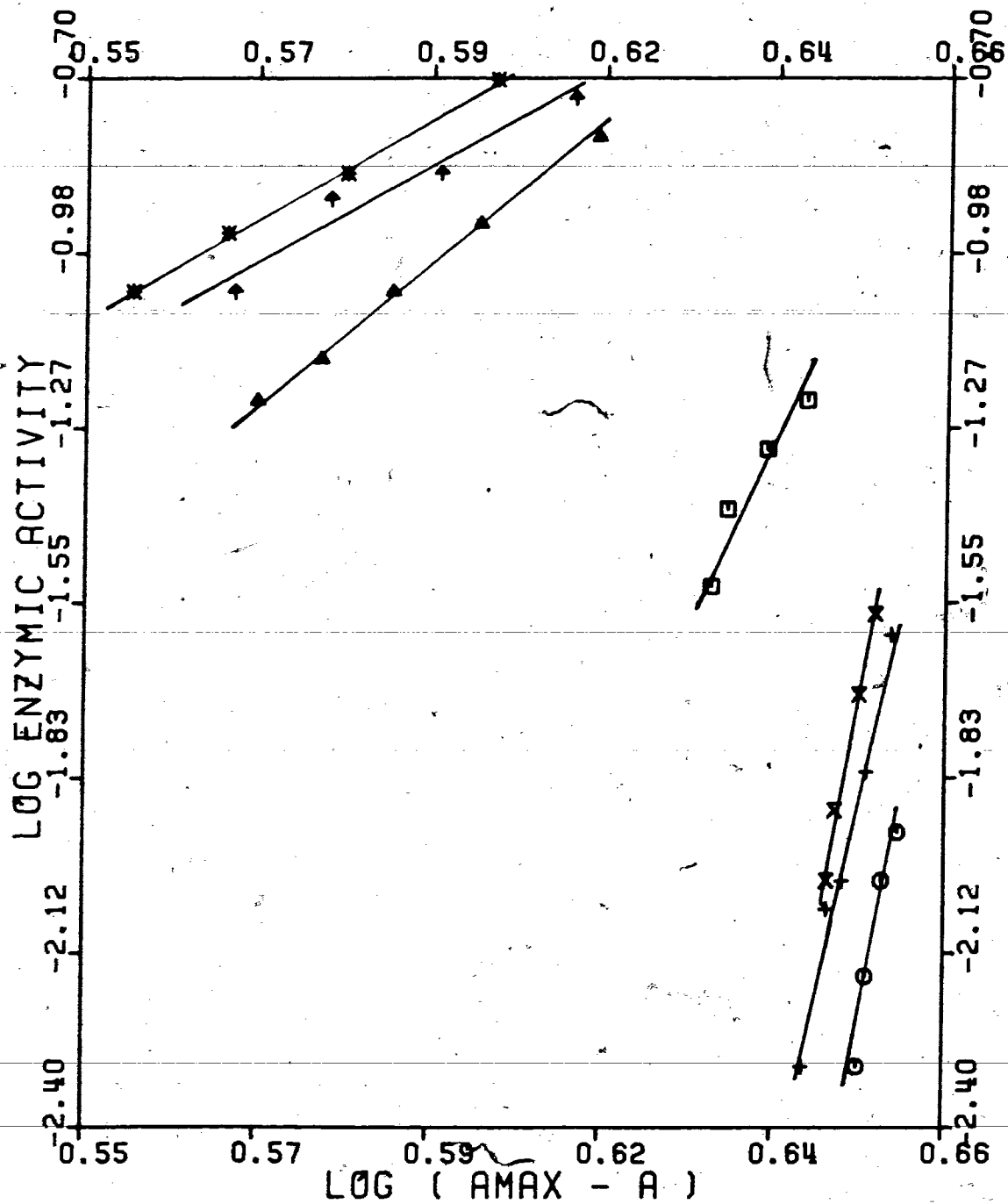
TABLE 1. EFFECT OF DTT ON THE RATE OF PRODUCT INHIBITION ON
MEMBRANE-BOUND G3PDH. (Amax-A)/t was plotted against
(Amax-A) for various concentrations of DTT. Amax is the
maximum absorbance at which NAD in the reaction mixture was
exhausted. A is the absorbance at time t. Conditions were
as described in Methods. 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.

concentrations of DTT (uM)	slopes (min-1)
20	60.4 +/- 4.6
30	73.8 +/- 13
40	69.3 +/- 4.5
50	23.2 +/- 4.7
60	6.8 +/- 1.1
80	10.1 +/- 0.3
100	7.4 +/- 0.2

BIBLIOGRAPHY

Cleland, W.W. 1963, Biochemistry, 3:480-482.
Dithiothreitol, a new protective reagent for sulphydryl
groups.

Cori, G.T., Slein, M.W., Cori, C.F.,1947, 605-618.
Crystalline D-glyceraldehyde-3-phosphate dehydrogenase from
rabbit muscle.

Furfine, C.S., Velick, S.F., 1965, J.Biol.Chem.,
240:844-855. The acyl-enzyme intermediate and the kinetic
mechanism of the glyceraldehyde-3-phosphate dehydrogenase.

Steck, T.L., Kant, J.A., 1974, Methods Enzymol., 31:172-180.
Preparation of impermeable ghosts and inside-out vesicles
from human erythrocyte membranes.

Tjioe, G., Haugaard, N., 1972, Life Sciences, 11:329-335.
Oxygen inhibition of the crystalline glyceraldehyde
phosphate dehydrogenase and disappearance of sulphydryl
groups.

BIBLIOGRAPHY

Adams, G.E., Wilson, R.L., Aldrich, J.E., Cundall, R.B., 1969, Int.J.Radiat.Biol., 16:333-342. On the mechanism of radiation-induced inactivation of lysozyme in dilute aqueous solution.

Adams, G.E., Wilson, R.L., Bisby, R.H., Cundall, R.B., 1971, Int.J.Radiat.Biol., 20:405-415. On the mechanism of the radiation-induced inactivation of ribonuclease in dilute aqueous solution.

Adams, G.E., Baverstock, K.F., Cundall, R.B., Redpath, J.L., 1973, Radiat.Res., 54:375-387. Radiation effects on alpha-chymotrypsin in aqueous solution: pulse radiolysis and inactivation studies.

Adams, G.E., Redpath, J.L., Bisby, R.H., and Cundall, R.B. 1972, Israel J. Chem. 10, 1079-1093. The use of free radical probes in the study of mechanisms of enzyme inactivation.

Adams, G.E., Boag, J.W., and Michael, B.D. 1966, Proc. Roy. Soc. (London) A289, 321. Transient species produced in irradiated water and aqueous solutions containing oxygen.

Adams, G.E., 1972, Adv.Radiat.Chem., 3:125-208. Radiation chemical mechanisms in radiation biology.

Alder, H.J., 1963, Radiat.Res.Suppl.3, p.110-129. Catalase, hydrogen peroxide and ionising radiation.

Alexander P. 1957, Radiat. Res. 6, 653-660. Effect of oxygen on inactivation of trypsin by the direct action of electrons and alpha particles.

Allen, A.O., 1961, The Radiation Chemistry of Water and Aqueous Solutions.   p.155.   D.Van Nostrand Comp., Inc.

Allen, D.W., Jandl, J.H., 1961, J.Clin.Invest., 6:454-475. Oxidative hemolysis and precipitation of hemoglobin.   II. Role of thiols in oxidant drug action.

Alper, T., 1971, Biophysical Aspects of Radiation Quality, p.171.   Vienna.

Alper, T., 1956, Radiat.Res., 5:573-586.   The modification of damage caused by primary ionisation of biological targets.

Alper, T., 1968, Nature (London), 217:862-863.   Low oxygen enhancement ratios for radiosensitive bacterial strains, and the probable interaction of two types of primary lesion.

Anbar, M., Neta, P., 1967, Int.J.Appl.Radiat.Isotopes, 18:493-523.   A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in aqueous solutions.

Augenstine, L.G.   1962, Adv. Enzymol.   24, 359-413. The effect of ionising radiation on enzymes.

Asbell, S.O. and Kramer, S.   1971, Radiology 98, 678-681.   Oxygen effect on production of radiation-induced myelitis in rats.

Bacq, Z.M. and Alexander, P.   In: Dickens, F., Oxygen in the Animal Organism.   1964, 509-535.

Bannister, J., Bannister, W., Wood, E., 1971, Eur.J.Biochm. 18:178-186.   Bovine erythrocyte cupro-zinc protein.   1. Isolation and general characterization.

Barber, D.J.W., Thomas, J.K., 1978, Radiat.Res., 74:51-65. Reactions of radicals with lecithin bilayers.

Beauchamp, C.O., Fridovich, I., 1970, J.Biol.Chem., 245:4641-4646. A mechanism for the production of ethylene from methional. The generation of .OH radical by xanthine oxidase.

Behar, D., Czapski, G., Rabani, J., Dorfman, L.M., Schwarz, H.A., 1970, J.Phys.Chem., 74:3209-3213. The acid dissociation constant and decay kinetics of the perhydroxyl radical.

Bielski, B.H.J., Richter, H.W., 1977, J.Am.Chem.Soc., 99:3019-3023. A study of O2- radical chemistry by stopped-flow radiolysis and radiation induced oxygen consumption.

Bisby, R.H., Cundall, R.B., Wardman, P., 1975, Biochim.Biophys.Acta., 389:137-144. A pulse radiolysis study of some free radical reactions with erythrocyte membranes.

Bland, J., Canfield, W., Kennedy, T., Vincent, J., Wells, R., 1978, Physiol.Chem. and Physics, 10:145-152. Effect of tocopherol on photooxidation rate of human erythrocyte membrane IN VITRO.

Bland, J., Madden, P., and Herbert, E.J., 1975, Physiol. Chem. and Physics, 7:69-75. Effect of alpha-tocopherol on the rate of photohaemolysis in human erythrocyte.

Bramley, T.A., Coleman, R., Finean, J.B., 1971, Biochim.Biophys.Acta, 241:752-769. Chemical, enzymological and permeability properties of human erythrocyte ghosts prepared by hypotonic lysis in media of different osmolarities.

Bramley, T.A., Coleman, R., 1972, Biochim.Biophys.Acta, 290:219-228. Effects of inclusion of Ca2+, Mg2+, EDTA or EGTA during the preparation of erythrocyte ghosts by hypotonic haemolysis.

Bray, R.C., 1970, Biochem.J., 117:13p. Free-radical intermediates in the reduction of oxygen.

Buchanan, J.D., Armstrong, D.A., 1978, Int.J.Radiat.Biol., 33:410-419. The radiolysis of glyceraldehyde-3-phosphate dehydrogenase.

Buettner, G.R., Oberley, L.W., Chan-Leuthauser, S.W.H., 1978, Photochem.Photcbiol., 28:693-695. The effect of iron on the distribution of superoxide and .OH radicals as seen by spin-trapping and on the superoxide dismutase assay.

Butler, J., and Robins, A. 1960, Nature 186, 697-698. Effect of oxygen on the inactivation of trypsin by ionising radiation.

Carter, J.R.jr., 1973, Biochm., 12:171-176. Role of sulphydryl in membrane structure.

Cleland, W.W. 1963, Biochemistry, 3:480-482. Dithiothreitol, a new protective reagent for sulphydryl groups.

Conway, A., Koshland, D.E., 1968, Biochem. N.Y., 7:4011-4023. Negative cooperativity in enzyme action. The binding of diphosphopyridine nucleotide to glyceraldehyde-3-phosphate dehydrogenase.

Coy, P. and Dolman, C.L. 1971, Brit. J. Radiology 44, 705-707. Radiation myelopathy in relation to oxygen level.

Cori, G.T., Slein, M.W., Cori, C.F.,1947, 605-618.
Crystalline D-glyceraldehyde-3-phosphate dehydrogenase from
rabbit muscle.

Cohen, G., Heikkela, R.E., 1974, J.Biochem., 249:2447-2452.
The generation of hydrogen peroxide, superoxide radical, and
hydroxyl radical by 6-hydroxydopamine, dialuric acid, and
related cytotoxic agents.

Czapski, G., Allen, A.O., 1962, J.Phys.Chem., 66:262-266.
Evidence for the superoxide-dependent reduction of Fe3+ and
its role in enzymatic generated .OH radical formation.

Dale, W.M., Gray, L.H., and Meredith. W. 1949, Phil.
Trans. Roy. Soc. London 242A, 33-62 Inactivation of
an enzyme (carboxypeptidase) by X- and alpha- radiation.

Dainton, F.S., Rowbottom, J., 1953, Trans.Farad.Soc.,
49:1160-1173. The primary radical yield in water: a
comparison of photolysis and radiolysis of H2O2.

Davison, A., Kong, S.K., 1977, Pacific Slope Biochemical
Conference, p.45. Free radical mechanisms in the effects of
gamma-irradiation on the plasma membranes of resealed
erythrocyte ghosts.

Davison, A.J. and Wainio, W.W. 1965, J. Biol.
Chem. 243, 5023-5027. Reactions of oxygenated
cytochrome oxidase.

Davison, A., Popma, A., 1978, Int.Congr.Nutrition, 10:146.
Effects of excess retinol, tocopherol, or ascorbate on lipid
autoxidation, enzyme inactivation and permeability of the
plasma membrane of erythrocyte ghosts.

Davison,A.J., Kaminsky, L.S., 1974, Proc.5th.Int.Hyperbaric
Congress (Vancouver), Trapp, W., Bannister, E., Davison, A.,
Trapp, P. (eds.), Simon Fraser Press. Involvement of
oxygen radicals in radiation damage.

Demopoulos, H.B., 1973a, Fed.Proc., 32:1859-1861 The basis of free radical pathology.

Demopoulos, H.B., 1973b, Fed.Proc., 32:1903-1908. Control of free radicals in biologic systems.

Dodge, J.T., Mitchell, C., Hanahan, D.J., 1963, Arch.Bioch.Biophys., 100:119-130. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes.

Draganic, I.G., Draganic, Z.D., 1969a, J.Phys.Chem., 73:2571-2577. On the origin of primary hydrogen peroxide yield in the gamma radiolysis of water.

Draganic, I.G., Nenadovic, M.T., Draganic, Z.D., 1969b, J.Phys.Chem., 73:2564-2571. Radiolysis of HCOOH + O2 at pH 1.3-13 and the yields, of primary products in gamma radiolysis of water.

Draganic, I.G., Draganic, Z.D., 1971, The Radiation Chemistry of Water. Academic Press.

Dragganic, Z.D., Draganic, I.G., 1971, J.Phys.Chem., 75:3950-3957. Studies of formation of primary yields of hydrogen peroxide and molecular hydrogen in the gamma radiolysis of neutral aqueous solutions.

Draganic, Z.D., Draganic, I.G., 1972, J.Phys.Chem., 1972, 76:2733-2737. Studies on the formation of primary hydrogen atom yield in the gamma radiolysis of water.

Draganic, Z.D., Draganic, I.G., 1973, J.Phys.Chem., 77:765-772. Studies on the formation of primary yields of hydroxyl radical and hydrated electron in the gamma radiolysis of water.

Duchon, G., Collier, B., 1971, J.Membr.Biol., 6:138-157.
Enzyme activities of human erythrocyte ghosts : effects of
various treatments.

Epp, E.R., Weiss, H., Djordjevic, B., and Santomasso,
A. 1972. Radiat. Res. 52, 321-332. The
radiosensitivity of cultured mammalian cells exposed to
single high intensity pulses of electrons in various
concentrations of oxygen.

Ewing, D., 1978, Radiat.Res., 73:121-136. Additive
sensitization of bacterial spores by oxygen and
p-nitroacetophenone.

Faraggi, M., Bettleheim, A., 1977, Radiat.Res., 72:81-88.
The reaction of the hydrated electron with amino acids,
peptides, and proteins in aqueous solutions.

Fee, J.A., Bergamini, R., Briggs, R., 1975,
Arch.Biochm.Biophys., 169:160-167. Observations on the
mechanism of the oxygen/dialuric acid-induced hemolysis of
vitamin E-deficient rat red blood cells and the protective
roles of catalase and superoxide dismutase.

Fee, J.A., Teitelbaum, H.D., 1972,
Bioch.Biophys.Res.Commun., 49:150-157. Evidence that
superoxide dismutase plays a role in protecting red blood
cells against peroxidative hemolysis.

Ferradini, C., Foos, J., Houee, C, Pucheault, J., 1978,
Photochem.Photobiol., 28:697-700. The reaction between
superoxide anions and H2O2.

Flossmann, W., Westhof, E., 1978, Radiat.Res., 73:75-85.
The action of ionising radiation on protein. II. Radical
formation in L-tryptophan-HCl.

Folkman, J., Conn, H., and Harmel, R.   1972, Science
180, 170-172. Acrylamide polymerisation: new method
for determining oxygen content in blood.

Folkman, J.   1972,.   J. ' Exptl.   Med.   133, 275
Measurement of oxygen content in blood and other liquids
by oxygen inhibition of chemical reactions.

Fong, K.I., McCay, P.B., Poyer, J.L., Misra, H.P., Keele,
B.B., 1976, Chem.Bicl.Interact., 15:77-89 Evidence for
superoxide-dependent reduction of Fe3+ and its role in
enzyme-generated .OH radical formation.

Fong, K.I., McCay, P.B., Poyer, J.L., Misra, H.P., Keele,
B.B., 1973, 248:7792-7797. Evidence that peroxidation of
lysosomal membranes is initiated by .OH free radicals
produced during flavin enzyme activity.

Fridovich, I., 1975, American Scientist, 63:54-59. Oxygen:
boon and bane.

Friedman, M., 1973, The chemistry and biochemistry of the
sulphydryl group in amino acids, peptides and proteins.
(Pergamon)

Frimer,A.A., Rosenthal, L., 1978, Photochem.Photcbicl.,
28:711-719. chemical reactions of superoxide anion radical
in aprotic solvents.

Purfine, C.S., Velick, S.F., 1965, J.Biol.Chem.,
240:844-855. The acyl-enzyme intermediate and the kinetic
mechanism cf the glyceraldehyde-3-phosphate dehydrogenase.

George, P., 1965, Oxidases and related redox systems, 1:3,
ed. by King, T.E., Mason, H.S., and Morrison, M., N.Y.,
John Wiley and Sons Inc.

Gilbert, D.   1972, Anesthesiol.   37, 100-111.   Oxygen
and life.

Goldstein, G.M., and Doull, J.   1972, Proc. Soc.
Exptl. Biol. Med.   138, 137-139.   Treatment of
nitrite-induced methemoglobinemia with hyperbaric
oxygen.

Goldstein, B.D., 1974, Brit.J.Haematol., 27:533.   Effects of
irradiation on red cells.

Goscin, S.A., Fridovich, I., 1972, Arch.Bioch.Biophys.,
153:778-783.   The role of superoxide radical in
non-enzymatic hydroxylation.

Gregory, E. and Fridovich, I.   1973, J. Bacteriol,
114, 1193-1197.   Oxygen toxicity and superoxide
dismutase.

Gray, L.H. and Scott, O.C.A. In: Dickens, P. Oxygen
in the animal organism.   1964, 537-553. Oxygen
tension and the radiosensitivity of tumours.

Haber,F., Weiss, J., 1934, Proc.R.Soc.London A, 147:332-351.
Catalytic decomposition of hydrogen peroxide by iron salts.

Halliwell, B., 1976, FEBS Lett. 72:8-10. An attempt to
demonstrate a reaction between superoxide and H_2O_2.

Halliwell, B., 1978, FEBS Lett., 96:238-242.
Superoxide-dependent formation of .OH radicals in the
presence of iron salts. Its role in degradation of
hyaluronic acid by a superoxide generating system.

Hanahan, D.J., 1973, Biochim.Biophys.Acta, 300:319-340.   The
erythrocyte membrane variability and membrane enzyme
activity.

Hanstein, W.G., Hatefi, Y., 1970, Arch.Bioch.Biophys., 138: 319-340. Lipid oxidation in biological membranes. II. Kinetics and mechanisms of lipid oxidation in submitochondrial particles.

Haugaard, N. 1968, Physiological Reviews. 48, 311-375. Cellular mechanisms of oxygen toxicity.

Hatefi, Y., Hanstein, W.G., 1970, Arch.Biochm.Biophys., 138:78-86. Lipid oxidation in biological membranes. I. Lipid oxidation submitcchondrial particles and microsomes induced by chaotrcpic agents.

Hendriksen, T. 1967, Radiat. Res. 32 892-904. Effect of oxygen on radiation-induced free radicals in proteins.

Herbert, D., Pinsent, J., 1948, Biochem.J., 43:2C3-205. Crystalline human erythrocyte catalase.

Hoffman, J.F., 1958, J.Gen.Physiol., 42:9-28. Physiological characteristics of human red blood cell ghosts.

Hodgson, E.K., Fridovich, I., 1976a, Biochim.Biophys.Acta, 430:182-188. The accumulation of superoxide radical during aerobic action of xanthine oxidase.

Hodgson, E.K., Fridcvich, I., 1976b, Arch.Bioch.Eiophys., 172:202-205. The mechanism of the activity dependent luminescence of xanthine oxidase.

Holmes, B. 1950, Nature 165, 266-269. Inactivation of ribonuclease in dilute aqueous soluticns.

Howard-Flanders, P., 1960, Nature, 186:485-487. Effect of oxygen on the radiosensitivity cf bacteriophage in the presence of sulphydryl compcunds.

Hutchinson, F.   1960, Radiat. Res. 14, 721-731. The effect of oxygen and some other gases on the radiation sensitivity cf dry trypsin.

Huyser, E.S., 1970, Free Radical Chain Reactions, Wiley-interscience.

Jacob, H.S., Jandl, J.H., 1962, J.Clin.Invest., 4:779-792. Effects of sulphydryl inhibition on red blood cells. I. Mechanism of hemolysis.

Jacob, H.S., Jandl, J.H., 1962, J.Clin.Invest., 7:1514-1523. Effects of sulphydryl inhibition on red blood cells. II. Studies IN VIVO.

Kaschnitz, R.M., Hatefi, Y., 1975, Arch.Biochem.Biophys., 171:292-304. Lipid oxidation in biological membranes.

Kellogg III, E.W., Fridovich, I., 1975, J.Biol.Chem., 250:8812-8817. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system.

Kellogg III, E.W., Fridovich, I., 1977, J.Biol.Chem., 252:6721-6728. Liposome oxidation and erythrocyte lysis by enzymatically generated superoxide and hydrogen peroxide.

Klebanoff, S.J., 1958, J.Gen.Physiol., 41:725-753. The effects of x-radiation on the glutathione metabolism of intact erythrocytes IN VITRO.

Koch, C.J., Kruuv, J., Frey, H.E., 1973, Radiat.Res., 53:33-42. Variation in radiation response of mammalian cells as a function cf oxygen tension.

Kong, S.K., M.sc. thesis, 1979, Simon Fraser University,
British Columbia, Canada. The effects of free radicals and
oxygen on the permeability and activity of
glyceraldehyde-3-phosphate dehydrogenase of resealed
erythrocyte ghosts.

Koppenol, W.H., Butler, J., 1977, FEBS Letter, 83:1-6. The
reactions involving singlet oxygen and superoxide anion.

Koppenol, W.H., Butler, J., vanLeenwen, J.W., 1978,
Photochem.Photobiol., 28:655-660. The Haber-Weiss cycle.

Koter, M., Leyko, W., 1973, Annals Med.Sect.Pol.Acadi.Sci.,
18:56-58. The effect of gamma-irradiation on ultrastructure
of human erythrocyte ghosts.

Lamola, A.A., Yamane, T., Trozzolo, A.M., 1973, Science,
179:1131-1133. Cholesterol hydroperoxide formation in red
cell membranes and photohemolysis in erythropoietic
protoporphyria.

Lichtin, N.N., Ogdan, J., Stein, G., 1972,
Biochim.Bicphys.Acta., 276:124-142. Fast consecutive
radical processes within the ribonuclease molecule in
aqueous solution. II. Reaction with .OH radicals and
hydrated electron.

Lin, W.S., Armstrong, D.A., 1978, Int.J.Radiat.Biol.,
33:231-243. Effects of superoxide dismutase, dithiothreitol
and formate ion on the inactivation of papain by hydroxyl
and superoxide radicals in air-saturated solutions.

Lynch, R.E., Fridovich, I., 1978a, J.Biol.Chem.,
253:4697-4699. Permeation of the erythrocyte stroma by
superoxide radical.

Lynch, R.E., Fridovich, I., 1978b, J.Biol.Chem., 253:1838-1845. Effects of syperoxide radical on the erythrocyte membrane.

Mason, H.S., 1965, A.Rev.Biochem., 34:595-634. Oxidases.

Masuda, T., Ovadia, J., Grossweiner, L.I., 1971, Int.J.Radiat.Biol., 20:447-459. The pulse radiolysis and inactivation of trypsin.

Mee, L.K. 1958. 26th Ann. Rept. British Empire Cancer Society, p.318.

McClune, G.J., Fee, J.A., 1976, FEBS Lett., 67:194-298. Stopped-flow spectrophotometric observation of superoxide dismutation in aqueous solution.

McCord, J.M., 1974, Sci., 185:529-531. Free radicals and inflammation: protection of syncvial fluid by superoxide dismutase.

McCord, J.M., Day, E.D.jr., 1978, FEBS Lett.,86:139-142. Superoxide-dependent production of .OH radical catalysed by iron EDTA complexes.

McCord, J.M., Keele, B.B. Jr., and Fridovich, I. Proc. Nat. Acad. Sci. U.S. 68, 1971, 1024-1027. An enzyme-based theory of obligate anaerobicsis: the physiological function of superoxide dismutase.

Mc Cord, J.M. and Fridovich, I. 1973, Photochem. Photobiol. 17; 115-121. Production of superoxide in photolyzed water demcnstrated through the use of superoxide dismutase.

McCord, J.M., Fridovich. I., 1969, J.Biol.Chem., 244:6049-6055. Superoxide dismutase, an enzymatic function for erythrocuprein.

Michaels, H.B., Hunt, J.W., 1977, Radiat.Res., 72:18-31. Reaction of oxygen with radiation-induced free radicals on single-stranded polynucleotides.

Milvy, P. 1973, Fed. Proc. 32, 1895-1902. Control of free radical mechanisms in nucleic acid systems: studies in radioprotection and radiosensitization.

Mittal, J.P., Hayon, E., 1974, J.Phys.Chem., 78:1790-1794. Interaction of hydrated electrons with phenylalanine and related compounds.

Myers, L.S. 1973, Fed. Proc. 32, 1882-1894. Free radical damage to nucleic acids and their components by ionising radiation.

Neta, P., Simic, M., Hayon, E., 1970, J.Phys.Chem., 74:1214-1220. Pulse radiolysis of aliphatic acids in aqueous solution. III. simple amino acids.

Nicholls, P., 1972, Biochim.Biophys.Acta., 279:306-309. Contribution of catalase and glutathione peroxidase to red cell peroxide removal.

Orr. C 1971, Biochemistry 6, 2995-3000. Studies on ascorbic acid. I. factors influencing the ascorbate-mediated inhibition of catalase.

Palcic, B. and Skarsgard, L.D. 1972, Int. J. Radiat. Biol. 21, 417-433. The effect of oxygen on DNA single strand breaks produced by ionising radiation in mammalian cells.

Petkau, A., Kelly, K., Chelack, W.S., Barefoot, C., 1976, Bioch.Bophys.Res.Comm., 70:452-458. Protective effect of superoxide dismutase on erythrocytes of X-irradiated mice.

Petkau, A., Chelack, W.S., 1976, Biochim.Biophys.Acta, 433:445-456. Radioprotective effect of superoxide dismutase on model phospholipid membranes.

Popma, M.A., M.sc. thesis, 1974, Simon Fraser University, British Columbia, Canada. The effects of retinol, alpha-tocopherol and ascorbic acid on the plasma membrane of erythrocyte ghosts.

Powers, E.L., 1972, Israel J.Chem., 10:1199-1211. The hydrated electron, the hydroxyl radical and hydrogen peroxide in radiation damage in cells.

Purdie, J.W., 1971, Radiat.Res., 48:474-483. Investigation of chain reactions and oxygen effects during radiolysis of peptide disulphide bonds using cysteine-glutathione disulphide as a model.

Pryor, W. 1973, Fed. Proc. 32, 1862-1869. Free-radical reactions and their importance in biochemical systems.

Quintiliani, M., Badiello, R., Tamba, M., Esfandi, A., Gorin, G., 1977, Int.J.Radiat.Biol., 32:195-202. Radiolysis of glutathione in oxygen-containing solutions of pH 7.

Rabani, J., Klug, D., Fridovich, I., 1972, Israel J.Chem., 10:1095-1106. Decay of the HO2 and O2- radicals catalysed by superoxide dismutase. A pulse radiolytic investigation.

Raleigh, J.A., Kremers, W., Gaboury, B., 1977, Int.J.Radiat.Biol., 31:203-213. Dose-rate and oxygen effects in models of lipid membranes: linoleic acid.

Rapoport, S.M., Muller, M., 1974, p.167-180, Catalase and Glutathione peroxidase, "Cellular and Molecular Biology of Erythrocytes" edited by Yoshikawa, H., and Rapoport, S.M., University Park Press, London.

Raymond, S. and Weintraub, L. 1952, Science 130, 711. Acrylamide gel as a supporting medium for zone electrophoresis.

Revesz, L. 1974. In: 5th International Hyperbaric Congress (Vancouver, Canada). Trapp, W., E. Bannister, A. Davison and P. Trapp (eds.) Simon Fraser University. Recovery process and the role of oxygen.

Robinson, J.D., 1965, Arch.Bioch.Biophys., 112:170-179. Structural changes in microsomal suspensions. III. Formation of lipid peroxides.

Robinson, J.D., 1966, Nature (London), 212:199-120. Interaction between sulphydryl groups and lipid double bonds in biological membranes.

Rodan, S.R., Rodan, G.A., Sha'afi, R.I., 1976, Biochim.Biophys.Acta, 428:509-515. Demonstration of adenylate cyclase activity in human red blood cell ghosts.

Rustgi, S., Joshi, A., Riesz, P., 1977, Int.J.Radiat.Biol., 32:533-552. E.s.r. of spin-trapped radicals in aqueous solutions of amino acids. Reaction of the hydrated electron.

Schrier, S.L., 1966, Am.J.Physiol., 210:139-145. Organisation of enzymes in human erythrocyte membranes.

Schrier, S.L., 1963, J.Clin.Invest., 42:756-766. Studies of the metabolism of human erythrocyte membranes.

Schwoch, G., Passow, H., 1973, Molecular and Cellular Biochemistry, 2:197-218. Preparation and properties of human erythrocyte ghosts.

Shanklin, D.R. 1969, Perspect. Biol. Med. 13, 80-100. Oxygen toxicity in man.

Shohet, S., 1972, New Eng. J. Med. 286, 577-644. Hemolysis and changes in erythrocyte membrane lipids.

Singh, A., 1978, Photochem.Photobiol., 28:429-433. Introduction: interconversion of singlet oxygen and related species. The International conference on singlet oxygen and related species in chemistry and biology.

Slater, T.F. 1972, Free radical mechanisms in tissue injury.(Pion Press, London).

Sokal, R.R., Rohlf, F.J., 1969, Biometry, the principles and practice of statistics in biological research, Freeman and Co., p.448-458.

Staros, J.V., Haley, B.E., Richards, F.M., 1974, J.Biol.Chem., 249:5004-5007 Human erythrocytes and resealed ghosts: a comparison of membrane topology.

Steck, T.L., Kant, J.A., 1974, Methods Enzymol., 31:172-180. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes.

Stocks, J., Dormandy, T.L., 1971, Brit.J.Haematol., 20:95-111. The autoxidation of human red cell lipids induced by H_2O_2.

Sutton, H.C., Roberts, P.B., Winterbourn, C.C., 1976, Biochem.J., 155:503-510. The rate of reaction of superoxide radical ion with oxyhaemoglobin and methaemoglobin.

Sutton, H.C.   1952.- Discussions Faraday Soc.   p.
281.

Svingen, B.A., O'Neal, F.O., and Aust, S.D., 1978,
Photochem.Photobiol., 28:803-809.   The role of superoxide
and singlet oxygen in lipid peroxidation.

Srivastava, B.S., 1974, Int.J.Radiat.Biol., 26:391-394.   The
yield of single-strand breaks in the DNA of bacteriophage
lamda irradiated extra- and intracellularly with gamma-ray
in oxic and anoxic conditions.

Swallow, A.J., Radiation Chemistry, 1973, John Wiley and
Sons, N.Y.

Tappel, A.L., Adhikari, H.R., 1975, Radiat.Res., 61:177-183.
Fluorescent products from irradiated amino acids and
proteins.

Tappel, A.L., 1973, Fed.Proc., 32:1870-1874.   Lipid
peroxidation damage to cell compcnents.

Taverna, R.D., Langdcn, R.G., 1973, Bicchim.Biophys.Acta,
298:422-428.   Glucose transport in white erythrocyte ghosts
and membrane-derived vesicles.

Teipel, J., Kcshland, D.E., 1970, Biochim.Biophys.Acta.,
198:183-191.   The effect of NAD+ on the catalytic efficiency
of glyceraldehyde-3-phosphate dehydrogenase from rabbit
muscle.

Tillmann, W., Cordua, A., Schroter, W., 1975,
Biochim.Biophys.Acta, 382:157-171.   Organisation of enzymes
of glycolysis and of glutathione metabolism in human red
cell membranes.

Tjioe, G., Haugaard, N., 1972, Life Sciences, 11:329-335.
Oxygen inhibition of the crystalline glyceraldehyde
phosphate dehydrogenase and disappearance of sulphydryl
groups.

Valentine .W. 1972, Ann. Rev. Med. 23, 93-100
Red-cell enzyme deficiencies as a cause of haemolytic
disorders.

Van der Schans, G.P., Blok, J., 1969, Int.J.Radiat.Biol.,
17:25-38. The influence of oxygen and sulphydryl compounds
on the production of breaks in bacteriophage DNA by
gamma-rays.

Van Hemmen, J.J., Meuling, W.J.A., Bleichrodt, J.F., 1978,
Radiat.Res., 75:410-423. Effect of oxygen on inactivation
of biologically active DNA by gamma rays IN VITRO :
influence of metalloporphyrins and enzymatic DNA repair.

Whittam, R., 1964, Transport and Diffusion in Red Blood
Cells. Edward Arnold Ltd., London, p.76-96.

Wills, E.D., Wilkinson, A.E., 1967, Radiat.Res., 31:732-747.
The effect of irradiation on lipid peroxide formation in
subcellular fractions.

Wills, E.D., Rotblat, J., 1965, Int.J.Radiat.Biol.,
8:551-567. The formation of peroxides in tissue lipids and
unsaturated fatty acids by irradiation.

Woodward, C.B., Zwaal, R.F.A., 1972, Biochim.Biophys.Acta.,
274:272-278. The lytic behaviour of pure phospholipases A2
and C towards osmotically swollen erythrocytes and resealed
ghosts.

Yonei, S., Todo, T., and Kato, M., 1979, Int.J.Radiat.Biol.,
35:161-170. Radiation effects on erythrocyte membrane
structure studied by the intrinsic fluorescence .

Yuhas, J.M., Li, A.P., 1978, Radiat.Res., 75:563-572. In vitro studies on the radioresistance of oxic and hypoxic cells in the presence of both radioprotective and radiosensitizing drugs.

Yung, C.Y., Carlson, L.M., Balzer, C.J., 1972, Biochim.Biophys.Acta, Characteristics of the permeability barrier of human erythrocyte ghosts to nonelectrolytes.

Zimmermann, R., Flohe, L., Weser, U., Hartmann, H.J., 1973, FEBS Letter, 29:117-120. Inhibition of of lipid peroxidation in isolated inner membrane of rat liver mitochondria by superoxide dismutase.

Zipursky, A., Stephens, M., Brown, E.J., Larsen, P., 1974, J.Clin.Invest., 53:805-812. Sulphydryl groups of the erythrocyte membrane and their relation to glycolysis and drug-induced hemolytic anaemia.

Zwaal, R.F.A., Roelofsen, B., Colley, C.M., 1973, Biochim.Biophys.Acta, 300:159-182 Localisation of red cell membrane constituents.