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THE RELATIVE EFFECTIVENESS OF ·OH, H_2O_2, O_2^-, AND REDUCING FREE RADICALS IN CAUSING DAMAGE TO BIOMEMBRANES

A STUDY OF RADIATION DAMAGE TO ERYTHROCYTE GHOSTS USING SELECTIVE FREE RADICAL SCAVENGERS

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Summary

The relative effectiveness of oxidizing (·OH, H_2O_2), ambivalent (O_2^-) and reducing free radicals (e^- and CO_2^-) in causing damage to membranes and membrane-bound glyceraldehyde-3-phosphate dehydrogenase of resealed erythrocyte ghosts has been determined. The rates of damage to membrane-bound glyceraldehyde-3-phosphate dehydrogenase (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 glyceraldehyde-3-phosphate dehydrogenase. Each radical was selectively isolated from the mixture produced during gamma-irradiation, using appropriate mixtures of scavengers such as catalase, superoxide dismutase and formate. ·OH, O_2^- and H_2O_2 were approximately equally effective in inactivating membrane-bound glyceraldehyde-3-phosphate dehydrogenase, while e^- and CO_2^- were the least effective. R(enz) values of O_2^- and H_2O_2 were 10-times and of ·OH 15-times that of e^-. R(mb) values were quite similar for e^- and H_2O_2 (about twice that of O_2^-), while that of ·OH was 3-times that of O_2^-. Hence, with respect to R(mb): ·OH > e^- = H_2O_2 > O_2^-, and with respect to R(enz): ·OH > O_2^- = H_2O_2 >> 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 the enzyme than to the membranes. Comparison between H_2O_2 added as a chemical reagent and H_2O_2 formed by irradiation showed that membranes and membrane-bound glyceraldehyde-3-phosphate dehydrogenase were relatively inert to reagent H_2O_2 but markedly susceptible to the latter.

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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 [1—5], revealing the susceptibility of aromatic and sulphur-containing amino acids towards these species. The rate constants of the reactions of free radicals with biologically important molecules have been well documented by Anbar and Neta [6]. However, there have been 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 [7,8]. In recent studies, Bisby et al. [9] 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} $M^{-1} \cdot s^{-1}$. However, no reaction with the disulphide linkages of proteins was observed, and the low reactivities of *N*-acetylneuraminic acid and *N*-acetylglucosamine 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 is the peptide bonds of surface proteins. Faraggi and Bettleheim [10] have shown that the carbonyl carbons of the peptide linkage of proteins are quite reactive towards e^-.

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 and Thomas [11] found that lecithin reacted efficiently with ·OH and competed with other intramembrane solutes. This may explain the absence of products of the reactions between ·OH and aromatic amino acids observed in the study of Bisby et al. [9]. Also, reactions between ·OH and solutes incorporated into the synthetic lipid bilayers indicated the freedom of diffusion of ·OH through the bilayers. The consequences of attack by ·OH on lecithin bilayers included: increased fluidity of the polar head-group region, increased viscosity of the hydrophobic region of the lecithin bilayers and appearance of water in the bilayers. In contrast, e^- reacted relatively slowly with lecithin, the rate constants being about 10^7 $M^{-1} \cdot s^{-1}$. The rate constants of the reactions of e^- with solutes were also decreased 1000-fold when the solutes were solubilised in the lipid bilayers. The result indicated low reactivity of e^- towards lipid reflecting the restricted diffusion imposed on e^- by the lipid layers.

O_2^- was reported to permeate stromal membranes of erythrocyte vesicles with surprising ease and to cause lysis of the membranes [12] which was inhibited by superoxide dismutase. Lipid peroxidation caused by O_2^- is catalysed by metal complexes [13], and both O_2^- and singlet oxygen have been shown to propagate the resulting chain reactions, and to be implicated in causing lysis of erythrocyte membranes [12,14—16]. A direct relationship between lipid peroxidation and lysis of erythrocyte ghosts has been demonstrated [17,18].

Studies of free radicals with the SH enzymes, glyceraldehyde-3-phosphate dehydrogenase and papain, suggested that oxidation of the SH groups at

the catalytic sites was responsible for the inactivation of the enzymes [19,20]. These SH groups possess marked reactivity towards electrophilic reagents [21]. Thus, O_2^- was found to be one of the most effective radicals in causing irreversible inactivation of the enzyme, while H_2O_2 oxidized the SH groups to sulphenic acid which could be re-reduced by dithiothreitol, thereby restoring the enzymic activity. Inactivation by the reducing radicals $^{\cdot}H$ and e^- did not appear to be appreciable.

The present study is an investigation of the effects of $^{\cdot}OH$, O_2^-, H_2O_2 and e^- in producing damage to the plasma membranes of erythrocyte ghosts, as assessed by the change in permeability of the resealed ghosts and the inactivation of membrane-bound glyceraldehyde-3-phosphate dehydrogenase.

The free radicals used were formed from the radiolysis of water by gamma-irradiation. Radiolysis of H_2O in the absence of O_2 produces the following primary free radicals [22,23]:

$$H_2O \rightarrow e^-, H^{\cdot}, {}^{\cdot}OH, H_2O_2 \qquad (1)$$

and their initial yields are: $G(e^-) = 2.8$, $G(H^{\cdot}) = 0.6$, $G(^{\cdot}OH) = 2.8$ and $G(H_2O_2) = 0.6$ [24—28], where G is the number of molecules or ions formed per 100 eV of energy absorbed.

In the presence of O_2, the following subsequent reactions occur, resulting in a variety of secondary radicals:

$$O_2 + e \rightarrow O_2^- \qquad k = 1.88 \cdot 10^{10}\ M^{-1} \cdot s^{-1} \qquad (2)$$

$$O_2 + {}^{\cdot}H \rightarrow HO_2 \qquad k = 1.90 \cdot 10^{10}\ M^{-1} \cdot s^{-1} \qquad (3)$$

$$HO_2^{\cdot} \rightarrow H^+ + O_2^- \qquad pK = 4.8\ [29] \qquad (4)$$

$$HCOO^- + {}^{\cdot}OH \rightarrow COO^- + H_2O \qquad k = 2.5 \cdot 10^9\ M^{-1} \cdot s^{-1} \qquad (5)$$

$$COO^- + O_2 \rightarrow CO_2 + O_2^- \qquad k = 2.4 \cdot 10^9\ M^{-1} \cdot s^{-1} \qquad (6)$$

The above rate constants were taken from the data of Anbar and Neta [6].

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 radical can be studied independently. The further question of the results of interactions between these free radical forms of O_2, whether cooperative or inhibitory, are reported elsewhere [30].

Materials and Methods

Chemicals. Glyceraldehyde 3-phosphate, dithiothreitol, catalase, Triton X-100 and β-NAD were supplied by Sigma Chemical Co. Sodium arsenate, sodium pyrophosphate and 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 [31]. The preparation has an activity of 2301 units/mg protein. Catalase obtained from Sigma has an activity of 2500 units/mg. Both enzymes were added to a final concentration of 20 μg/ml, i.e., 46 units superoxide dismutase and 50 units catalase per ml solution. These concentrations were confirmed to be sufficient to scavenge over 90% of the O_2^- and H_2O_2 generated during irradiation [32,33] at the dose rate used throughout.

Ghost preparation. Resealed ghosts were prepared according to the method of Steck and Kant [34] with minor modifications as previously described [35]. We have estimated the concentration of residual haemoglobin to be 10% ± 5% [17]. The activities of residual catalase and dismutase were estimated to be 10^4 and 37 units per ml of packed ghosts, respectively, which are 12 and 8.7% of the activities in red blood cells.

Determination of the activity of membrane-bound glyceraldehyde-3-phosphate dehydrogenase and the permeability of the plasma membrane. Glyceraldehyde-3-phosphate dehydrogenase was assayed according to the method of Steck and Kant [34] except that dithiothreitol was used instead of cysteine hydrochloride. Membrane permeability was determined by measuring the accessibility of membrane-bound glyceraldehyde-3-phosphate dehydrogenase to its externally added substrates [34]. In the presence of dithiothreitol, the inhibition of glyceraldehyde-3-phosphate dehydrogenase by Triton X-100 was sufficiently small so that it did not affect the sensitivity of the assays. The percentage of active enzyme in 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, as described elsewhere [35].

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

Induction of atmospheres other than air. Buffers for incubation were carefully saturated with air, N_2 or O_2 before irradiation. However, since the membranes were air-equilibrated before introduction into the anaerobic media, 'anaerobic' in these experiments implies a residual O_2 concentration of about 23 μM which diminishes progressively to 0 μM after 14 min of irradiation.

Analysis of data. Slopes of the graphs in Figs. 1—4 were obtained by regression analysis, each line representing 18 experimental observations from three separate experiments. The standard errors of the slopes of graphs of permeability as a function of dose are on the average less than ±30% while those of enzymic inactivation are less than ±20%. The asymmetry of the gamma source and the inevitable variations in the membrane preparations are probably the major sources of these errors.

Production of O_2^-. e^- and $^{\cdot}H$ produced in reaction 1 react rapidly with O_2, and are converted to O_2^- by reactions 2—4. Using catalase to scavenge H_2O_2 [36] (reaction 7) and formate to scavenge $^{\cdot}OH$ [25] (reactions 5 and 6), irradiation of air-saturated aqueous solution produces predominantly O_2^-. The total yield of O_2^- is $G(O_2^-) = G(e^-) + G(H^{\cdot}) + G(^{\cdot}OH) = 6.2$

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2 \qquad k = 3.4 \cdot 10^7\ M^{-1} \cdot s^{-1} \tag{7}$$

Production of H_2O_2. If superoxide dismutase was used instead of catalase in the above solution, the O_2^- produced was converted to H_2O_2 according to reaction 8 [37]:

$$2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2 \qquad k = 2 \cdot 10^9\ M^{-1} \cdot s^{-1} \tag{8}$$

Therefore, $G(H_2O_2) = G(H_2O_2) + 0.5 \cdot G(O_2^-) = 0.6 + 3.1 = 3.7$.

Production of reducing radicals. Formate added to N_2-saturated solution removes ·OH as well as ·H, but formate is relatively less reactive towards e^- ($k(HCOO^- + e^-) < 10^{-6}\ M^{-1} \cdot s^{-1}$).

$$\cdot H + HCOO^- \rightarrow H_2 + COO^- \qquad k = 2.5 \cdot 10^8\ M^{-1} \cdot s^{-1} \tag{9}$$

Carboxyl radicals will be produced from reactions 5 and 9. Thus, the major radicals produced anaerobically are e^- ($G = 2.8$), CO_2^- ($G = 3.4$) and H_2O_2 ($G = 0.6$).

Production of ·OH. ·OH radicals can be isolated in air-saturated buffer by adding both catalase and dismutase to the medium.

Results

Calculation of R(mb) and R(enz)

Fig. 1 is a graph of the permeability of membranes as a function of dose. The slopes of the curves in Fig. 1 represent the rates at which ·OH, O_2^-, H_2O_2 and reducing radicals (e^- plus CO_2^-) caused increased permeability of the membrane. Comparison of the rates obtained from the slopes of the graphs does not directly indicate the effectiveness of each kind of radical in inducing

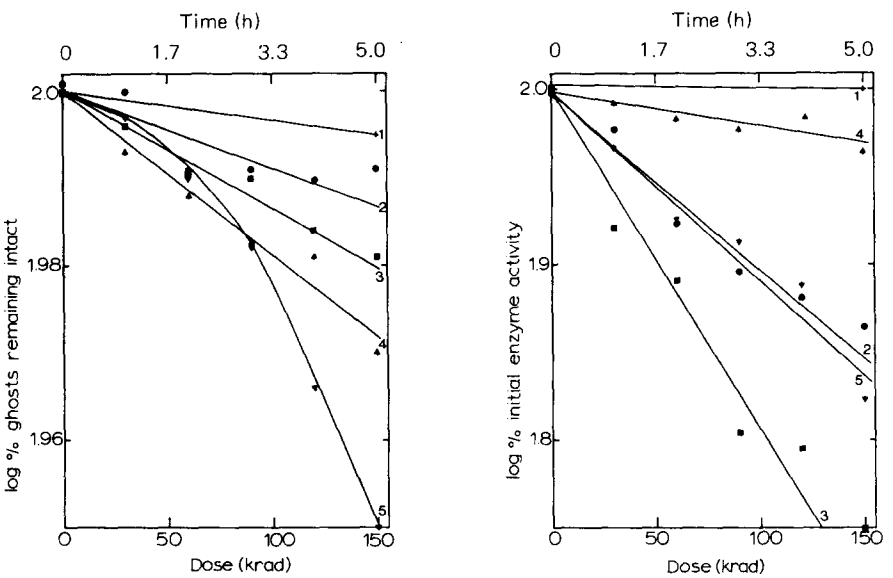

Fig. 1. Comparison of the effects of O_2^-, H_2O_2, ·OH and e^- on the permeability of plasma membranes of ghosts. Log% ghosts remaining intact per krad of irradiation is plotted against dose in krad. Conditions were: 0.01 M phosphate buffer, pH 7.4, rendered isotonic by the addition of NaCl to 300 mosM, temperature 25°C, concentration of ghosts or erythrocytes 10% (v/v), dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffered solutions saturated by atmospheric air unless O_2 or N_2 is specified. Catalase, superoxide dismutase or formate when present were at final concentrations of 50 units/ml, 46 units/ml and 10 mM, respectively, in the buffer solutions. The slopes and their standard errors were obtained by regression analysis as described in Materials and Methods. Control (+), ·OH (▼), O_2^- (■), H_2O_2 (●), $H_2O_2 + e^- + CO_2^-$ (▲).

Fig. 2. Comparison of the effects of O_2^-, H_2O_2, ·OH and e^- on the activity of membrane-bound glyceraldehyde-3-phosphate dehydrogenase of ghosts. Log% activity remaining per krad of irradiation is plotted against dose in krad. Conditions were as described in Fig. 1. Control (+), ·OH (▼), O_2^- (■), H_2O_2 (●), $H_2O_2 + e^- + CO_2^-$ (▲).

damage due to the different quantities of radicals produced under the different conditions. In order to compare the effectiveness of each kind of radical in causing damage, the ratio, R(mb), of the slope to the yield, G, of each kind of radical was used. Similarly, R(enz) was calculated for inactivation of glyceraldehyde-3-phosphate dehydrogenase by each kind of radical. These R values (Table II) represent the product of two characteristics or the effective destructive power of the radical, namely the reactivity of the radical and the probability that one hit of the radical would produce damage.

There is some ambiguity in the calculated R(mb) values for the reducing radicals: $1.0 \cdot 10^{-3} > R(\text{mb}) > 0.8 \cdot 10^{-3}$, depending on what fraction of the H_2O_2 produced is assumed to be consumed by reactions with e^- and CO_2^-. Similarly, R(enz) can be shown to be: $1.1 \cdot 10^{-3} > R(\text{enz}) > 0.3 \cdot 10^{-3}$.

Although the effects of these radicals were measured in the presence of O_2 at about 230 μM (air-saturated), acceleration of damage by O_2 resulting in an autocatalytic character of the kinetics of destruction was noted only in the presence of 100% O_2 [30].

Effects of free radicals on activity of membrane-bound glyceraldehyde-3-phosphate dehydrogenase and on permeability of membranes

The effects of individual free radicals on permeability and enzymic inactivation are shown in Figs. 1 and 2, respectively. In both figures, unirradiated ghosts were used as controls. Table I summarizes the yields of free radicals produced under different conditions and the gradients of the corresponding curves plotted in Figs. 1 and 2. The R values for individual radicals are compared in Table II. The R values show that while $^{\cdot}OH$ is the most destructive free radical towards both membranes and glyceraldehyde-3-phosphate dehydro-

TABLE I

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

The yields of free radicals are given in molecules or ions per 100 eV of radiation. The rate of damage to the membranes and the activity of glyceraldehyde-3-phosphate dehydrogenase were calculated from the slopes of Figs. 1 and 2. The rates are expressed in log% ghosts remaining intact and log% activity remaining per krad of dose. Conditions were: 0.01 M phosphate buffer, pH 7.4, rendered isotonic by the addition of NaCl to 300 mosM, temperature 25°C, concentration of ghosts or erythrocytes 10% (v/v), dose rate of gamma-irradiation 0.5 krad/min. Irradiation was carried out in buffered solutions saturated by atmospheric air unless O_2 or N_2 is specified. Catalase, superoxide dismutase or formate when present were at final concentrations of 50 units/ml, 46 units/ml and 10 mM, respectively, in the buffer solutions. The slopes and their standard errors were obtained by regression analysis (see Materials and Methods). The standard errors of most of the slopes of the plots of permeability and enzymic inactivation are less than ±30 and ±20%, respectively.

Scavengers	Radicals produced				Rate of increase in permeability ($\times 10^{-3}$)	Rate of inactivation of glyceraldehyde-3-phosphate dehydrogenase ($\times 10^{-3}$)
	H_2O_2	O_2^-	e^-	CO_2^-		
Formate, dismutase	3.7				3.5 ± 1.6	29.7 ± 3.9
Formate, catalase		6.2			3.9 ± 1.1	53.9 ± 8.7
N_2, formate	0.6		2.8	3.4	5.4 ± 1.3	6.4 ± 2.3
Catalase, dismutase	2.8				5.1 ± 1.4	32.5 ± 3.7

TABLE II

THE EFFECTIVENESS OF H_2O_2, O_2^-, e^- AND $^{\cdot}OH$ IN CAUSING DAMAGE TO PLASMA MEMBRANE AND MEMBRANE-BOUND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

R(mb) and R(enz) values were obtained by dividing the rates in Table I by the yield of the corresponding free radical in molecules or ions per 100 eV. Conditions were as described in Table I.

Radical	R(mb) ($\times 10^{-3}$)	R(enz) ($\times 10^{-3}$)
H_2O_2	1.0 ± 0.57	8.0 ± 1.4
O_2^-	0.6 ± 0.2	8.7 ± 1.8
$e^- + CO_2^-$	>0.8 ± 0.2	>0.3 ± 0.1
	<1.0 ± 0.3	<1.1 ± 0.5
$^{\cdot}OH$	1.8 ± 0.4	12.0 ± 1.2

genase, the oxidizing radicals, H_2O_2 or O_2^-, are more destructive than reducing radicals towards glyceraldehyde-3-phosphate dehydrogenase. In contrast, O_2^- is least destructive to membranes. R(enz) values of O_2^- and H_2O_2 were 12-fold and of $^{\cdot}OH$ 17-fold that of e^-, while R(mb) values of e^- and H_2O_2 were about 1.5-fold and of $^{\cdot}OH$ 3-fold that of O_2^-. Therefore, the difference between the effectiveness of the most damaging and the least damaging free radicals was at least 10-fold greater in damage to the enzyme than to the membranes. In the order of decreasing R(mb) values: $^{\cdot}OH > e^- = H_2O_2 > O_2^-$ and in the order of decreasing R(enz) values: $^{\cdot}OH >> O_2^- = H_2O_2 >> e^-$.

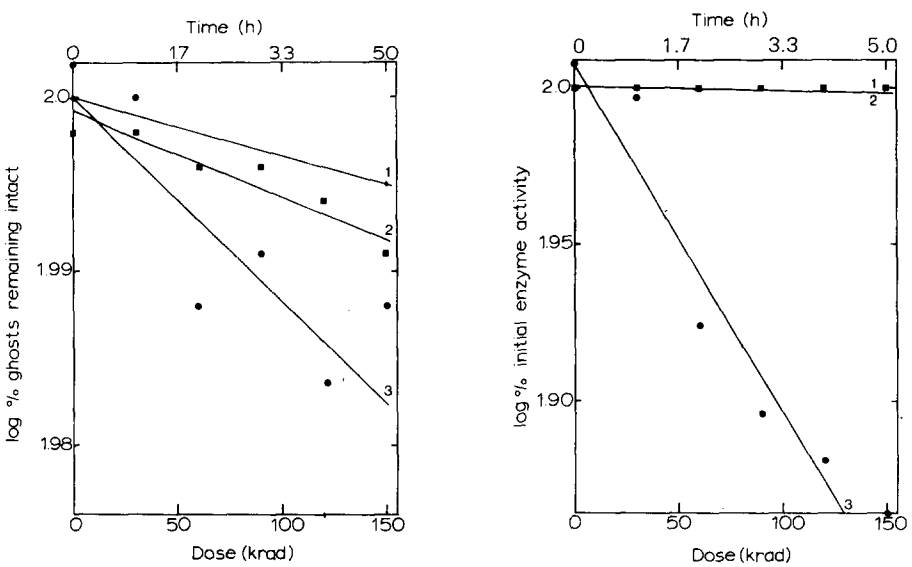

Fig. 3. Comparison of the effect of reagent H_2O_2 and radiation-induced H_2O_2 on the permeability of membranes. Log% ghosts remaining intact is plotted against both dose in krad and time in h. Conditions were as described in Fig. 1. Control (+), radiation-induced H_2O_2 (•), reagent H_2O_2 (▪).

Fig. 4. Comparison of the effect of reagent H_2O_2 and radiation-induced H_2O_2 on the activity of membrane-bound glyceraldehyde-3-phosphate dehydrogenase. Log% activity remaining is plotted against both dose in krad and time in h. Conditions were as described in Fig. 1. Control (+), radiation-induced H_2O_2 (•), reagent H_2O_2 (▪).

Comparison of reagent H_2O_2 with H_2O_2 produced by irradiation

Reagent H_2O_2 was added to the ghost suspension to a final concentration of 120 μM. Comparison of the damage due to reagent H_2O_2 with that due to H_2O_2 produced by irradiation in the presence of formate plus superoxide dismutase indicated a striking difference (Figs. 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.

Discussion

$^{\cdot}OH$, H_2O_2, O_2^- and e^- produced in the radiolysis of water were more destructive to membrane-bound glyceraldehyde-3-phosphate dehydrogenase than to membrane permeability by an order of magnitude, despite the relative inaccessibility of glyceraldehyde-3-phosphate dehydrogenase. This is due largely to the polar, hydrophilic character of these radicals which effectively excludes their presence from the water-deficient lipid bilayers. Furthermore, several neighbouring sites of damage may be required to produce an observable increase in permeability.

The greater inactivation of glyceraldehyde-3-phosphate dehydrogenase by radicals with high redox potentials indicates that oxidative damage may be the major cause of inactivation, consistent with the finding that the oxidation of the sulfhydryl group at the catalytic site of the enzyme is involved [19,20].

Formate was the only scavenger used that could diffuse through the membrane of erythrocytes [38]. Superoxide dismutase and catalase could 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^- [39], allowing these radicals to 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. It has been shown, for example, that externally added superoxide dismutase can effectively inhibit lysis of erythrocyte vesicles by O_2^- generated inside the vesicles [12]. Furthermore, residual catalase and dismutase in the ghosts would remove most of the free radicals formed inside.

Effects of O_2^-

Although damage to erythrocyte vesicles by O_2^- has been reported [12,40], O_2^- (when it is isolated from H_2O_2) is the least effective of the free radicals studied in causing lysis of the ghost membranes. The mechanism by which O_2^- increases permeability is unknown. Although its reactivity towards alkenes is low [41—43], complexed metals are known to catalyse lipid auto-oxidation by O_2^- [13]. Also, the reaction of O_2^- with membrane SH groups is rapid [44,45]. The low R(mb) value is due partly to the negative charge of O_2^-, which hinders its diffusion into the hydrophobic region of the plasma membrane. Its reaction is thus primarily with the exposed components of the membrane.

In contrast, O_2^- was one of the most strongly inactivating radicals towards

membrane-bound glyceraldehyde-3-phosphate dehydrogenase. It has been shown that O_2^- was unusually reactive towards the SH groups in the active sites of pure glyceraldehyde-3-phosphate dehydrogenase [46,47], and inactivated the enzyme via the sequence of reactions proposed by Lin and Armstrong [20].

Although the amount of O_2^- produced in the bulk of the medium is significantly greater than that produced inside the ghosts, and although residual superoxide dismutase is present in the ghosts, O_2^- is effective in inactivating glyceraldehyde-3-phosphate dehydrogenase for three reasons. First, resealed ghosts are permeable to O_2^- [39]. Second, its low reactivity towards membranes allows much of the O_2^- that permeates the membrane to react with glyceraldehyde-3-phosphate dehydrogenase. Third, although the residual superoxide dismutase in the ghosts removes O_2^- produced on the cytoplasmic side of the membrane, extraneous O_2^- reaching an element of volume adjacent to the cytoplasmic side of the membrane would not be effectively removed by the residual enzyme, due to the electrostatic repulsion between the membrane and the negatively charged dismutase [12], its isoelectric point being 4.95 [48].

Effects of hydrated e^-

From our data, we cannot separate the effects of e^- and CO_2^-, since they were produced together. However, most of the damage in the presence of N_2 plus formate can be attributed to e^- in view of the relatively low reactivity of CO_2^- [49], and the relatively small amounts of H_2O_2 produced (Table I).

The sphere of water around the hydrated e^- restricts its freedom to traverse the lipid bilayers. Even if it can pass through anion channels like O_2^-, its relatively higher reactivity would cause most of it to be consumed in reactions within the channels. Its access to the proteins bound on the cytoplasmic side or within the membrane is therefore limited. Hence, the membrane protects membrane-bound glyceraldehyde-3-phosphate dehydrogenase from e^-. Results from Bisby et al. [9] and Barber and Thomas [11] support this explanation. The latter workers found a 1000-fold decrease in the rate constants of the reactions of e^- due to the lipid layer and the former workers confirmed its low reactivity towards membranous SH groups of erythrocyte ghosts.

The moderate damage to membrane permeability caused by e^- represents a balance between its high reactivity and its restricted access to lipids due to its hydrophilic character. Carbonyl groups in the peptide bonds of surface proteins comprise one target site. The main consequence of this interaction is reductive deamination [10,50—53], leading to peptide breakage and release of free radicals which in turn can induce lipid autooxidation [54].

$$e^- + {}^+NH_3\text{-}CHR\text{-}CONH_2 \rightarrow {}^\cdot CHR\text{-}CONH_2 + NH_3 \qquad (10)$$

Effects of H_2O_2

H_2O_2 was found to cause reversible damage to pure glyceraldehyde-3-phosphate dehydrogenase and to papain [19,20] by oxidizing the enzymic SH group to sulphenic acid. However, our data revealed irreversible inactivation because incubation with dithiothreitol did not restore enzyme activity. The residual catalase found in our ghosts is 12% of that of red blood cells, and

unlike superoxide dismutase, catalase has a higher isoelectric point (pH 5.7). It is correspondingly more effective in protecting membrane-bound glyceraldehyde-3-phosphate dehydrogenase. Thus, the observed damage to glyceraldehyde-3-phosphate dehydrogenase requires some other explanation. Presumably damage was due to some reactive species, perhaps lipid peroxides, arising from the interaction of H_2O_2 with the membranes which could not be removed by catalase. Catalase added externally protects both membrane and membrane-bound glyceraldehyde-3-phosphate dehydrogenase [30], thus indicating that H_2O_2 generated externally was responsible for initiating the observed damage.

The difference in reactivity towards cellular constituents between reagent H_2O_2, and H_2O_2 produced by irradiation has been observed by Klebanoff [55]. 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 H_2O_2 produced by irradiation. Alder [56] found that H_2O_2, which had been generated enzymically, caused damage to cells comparable to that of H_2O_2 produced by irradiation. Obviously, in the presence of traces of catalase, a continous low level of H_2O_2 will be more damaging than a large initial dose which is not replaced after disproportionation.

Effects of ·OH

The high R(mb) value for ·OH could be explained by a combination of its high reactivity towards lipids (rate constant for reaction with phosphatidylcholine is $5.1 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) [11], and its solubility in the hydrophobic region of the membrane. The cross-linking between lipids and the increase in fluidity of the polar head-group 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 allowing radiolysis of water previously excluded from the mid-zone of membranes, and so accelerated damage. Also, cholesterol, which exists in equimolar proportions with phospholipids in the erythrocyte membrane, is known to form hydroperoxides with ·OH [57]. The hydroperoxides disrupt packing of lipids in the membrane, leading to leakiness of the membrane [58,59], in addition to triggering autocatalytic lipid auto-oxidation. In the presence of O_2, damage to membranes is dramatically accelerated, since O_2 participates in free radical chain reactions in the hydrophobic region. 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 O_2, they form hydroperoxides [35].

These two effects undoubtedly contribute to the acceleration of damage to membranes which resulted in the curvature of plots in Fig. 1, and account for the fact that this phenomenom is so pronounced in the presence of high concentrations of O_2.

The common features of O_2^-, H_2O_2 and ·OH that enable them to produce damage to membrane-bound glyceraldehyde-3-phosphate dehydrogenase are the permeability of the membranes to these radicals and their high redox potentials. The higher R(enz) value for ·OH is attributable to its higher redox potential. Bray [60] has reported a value of +2.3 V for the ·OH/H_2O pair. This

value, compared to +1.1 and +0.4 V for O_2^- and H_2O_2 [61,62], respectively, is in agreement with the order of the R(enz) values of these radicals.

The factors governing the effectiveness of a free radical in causing damage to membrane permeability and membrane-bound glyceraldehyde-3-phosphate dehydrogenase can be summarized:

(a) The reactivity of the radical towards specific components of membrane. For instance, the effectiveness of the oxidative free radicals correlates with their reactivity towards SH groups at the active site of membrane-bound glyceraldehyde-3-phosphate dehydrogenase and the reactivity of ·OH towards lecithin of membranes.

(b) Accessibility of membrane components to a given radical. This includes permeability characteristics of the membrane to the radical as well as the extent of exposure of the reactive groups of the component. Thus, the permeability of the ghost membrane to O_2^- allowed attack on SH groups on the cytoplasmic side of the membrane, while e^-, though more reactive, was diminished in effectiveness due to its restricted diffusion. Also, initial damage to the membrane alters the accessibility of its susceptible sites. Thus, conformational changes of damaged membrane proteins may produce deleterious effects on packing of lipid bilayers [63], and such changes also render membrane lipids as well as the SH groups and aromatic residues in the intramembranous proteins more accessible to radical attack through the resulting aqueous channels [44,45,64].

(c) 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 lipids is a major determinant of the relatively little damage to the membranes caused by these radicals.

(d) Protective reactions of the radical with other components, the alteration of which produces only minor effects, and which can be considered intrinsic nonspecific scavengers in the membrane. The surprisingly moderate damage by e^- to membranes as compared to its high reactivity illustrates this. Most of the e^- which attempts to diffuse through the membrane via aqueous channels reacts with channel proteins and very little thus reaches the cytoplasmic side. In effect, the lipids of the membrane protect glyceraldehyde-3-phosphate dehydrogenase against hydrated e^- but not against the other, more permeable, free radicals.

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