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DOES OXYGEN ENHANCE THE RADIATION - INDUCED INACTIVATION OF PENICILLINASE?

PROGRESS REPORT

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Work carried out during the period:
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ABSTRACT

The radiation-induced inactivation of penicillinase (β -lactamase EC 3.5.2.6.) in dilute aqueous solutions buffered with phosphate was studied, by examining enzyme radiosensitivity in the presence of various gases (He , O_2 , H_2 , N_2O , $\text{N}_2\text{O} + \text{O}_2$). The introduction of either N_2O or O_2 was found to reduce the radiodamage. On the other hand H_2 or $\text{N}_2\text{O} + \text{O}_2$ gas-mixture enhanced the radiosensitivity. In the presence of formate and oxygen no enzyme inactivation was detected. The results indicated that the specific damaging efficiency of $\dot{\text{H}}$ atoms is almost four-fold higher than that of $\dot{\text{OH}}$ radical; therefore in phosphate buffer, where more than half of the free radicals are $\dot{\text{H}}$ atoms, it is the $\dot{\text{H}}$ radicals that are responsible for the majority of the damage. The superoxide radicals appeared to be completely inactive and did not contribute toward enzyme inactivation. Oxygen was shown to affect the radiosensitivity in two ways. On one side, it protected by converting e_{aq}^- and $\dot{\text{H}}$ into harmless $\dot{\text{O}}_2^-$ radicals. On the other side it increased the inactivation by enhancing the damage brought about by $\dot{\text{OH}}$ radicals (OER = 2.8). In the present case the oxygen effect of protection exceeded that of sensitization, thus giving rise to a moderate overall protection effect.

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Key Words: γ -irradiation, oxygen effect, superoxide radicals, penicillinase, β -lactamase.

INTRODUCTION

The study of the effect of radiation on enzymes is of interest because they are essential for the maintenance of the vital processes. In addition, an investigation of isolated enzymes enables better understanding of the radiation effects at the molecular level. The influence of radiation on dilute enzyme solutions is mainly an *indirect* effect in which the radio-damage is brought about *via* the water-radicals. This and the lack of repair mechanisms operating within the test-system may assist in the elucidation of the roles of the damaging species. Therefore many enzymes have been used as test-systems for the study of the oxygen effect in radiodamage, yet the role of oxygen in those systems has not been satisfactorily elucidated.

Generally, very little or no enhancement of radiosensitivity due to oxygen was observed, when enzymes were irradiated in dilute aqueous solutions ((1-3), and references therein). In many cases the effect of oxygen was found to be a protective one (1-6). Sensitization, when found, was mainly for SH containing enzymes. The sensitizing effect of O_2 was ascribed to the conversion of the H and e^-_{aq} , by oxygen, into superoxide radicals (7-9). The same argument was adopted to explain the protective effect (10-11). It was also noted that sensitization by O_2 particularly appears in the presence of radioprotectants (1-3, 12). This observation was attributed to a possible competition of oxygen with chemical restitution processes (1, 12).

Since all of the evidence so far accumulated indicates that oxygen might modify the damage both by changing the spectrum of the water radicals and by interacting with the damaged biomolecules, it appeared to us useful to distinguish between these different functions. The present study attempts to make this distinction.

MATERIALS AND METHODS

Materials

Crystalline preparation of benzylpenicillin was obtained from Rafa Laboratories, Israel, and sodium formate from B.D.H. The enzyme employed was the extracellular preparation of penicillinase (EC 3.5.2.6.) derived from *Bacillus cereus* strain 569/H and purified as previously described (13). Enzyme solutions (0.2 - 2 μ M), buffered with 50mM phosphate pH 7, were prepared in triply distilled water immediately before irradiation. Organic buffering systems (like Tris) were avoided, as they react with the H and OH radicals formed. Deoxygenation, when required, was carried out by bubbling helium for 20 min through the enzyme solution, prior to irradiation. The displacing gases were first passed through a series of oxygen-traps (V^{++} , HCl, Hg/Zn), to remove traces of oxygen. Control experiments showed that the bubbling by itself did not reduce the enzymatic activity.

Enzyme Assay

To determine the enzymatic activity, the hydrolytic cleavage of the β -lactam ring of benzylpenicillin was assayed iodometrically(14) or spectrophotometrically at 240 nm (15). The unit of activity taken was the amount of enzyme required to catalyze the hydrolysis of 1 μ mole substrate in 1 hour at 30°C, pH 7.

γ -Irradiation

The enzyme solutions were irradiated in glass syringes, using a model M38-3 Gammator 2400 ci ^{137}Cs gamma source (Radiation Machinery Co., U.S.A.) The dose-rate employed was $1.7 \text{ krad min}^{-1}$, as determined by ferrous sulphate dosimetry. Irradiations were performed at room temperature ($22^{\circ}\text{C} \pm 2^{\circ}$).

RESULTS

Penicillinase Inactivation

Solutions of penicillinase buffered with 50mM phosphate pH 7, were γ -irradiated and the catalytic activity towards benzylpenicillin was determined. The dependence of the residual enzymatic activity on dose appeared to be exponential, as is generally found for isolated enzymes (16). In Fig. 1 the dose response curve obtained for penicillinase irradiated under helium is presented. Repetition of the experiment with various enzyme concentrations (0.2 - $2\mu\text{M}$) showed that the inactivation constant, evaluated from the plot, was inversely dependent on the concentration of the enzyme (increasing as expected with $1/[\text{enzyme}]$). Hence, comparison was made only for experiments that were carried out using the same initial enzyme concentration. The $G_{(\text{enzyme inactivation})}$ calculated from the initial slope amounted only to 0.055, indicating that only a small fraction of the water-radicals formed contributed to enzyme inactivation.

Effect of O_2 and H_2

To compare the radiosensitivity of penicillinase in phosphate buffer under O_2 and under H_2 , the enzyme solutions were bubbled with the respective gas prior to irradiation. The dose response curves obtained are presented in Fig. 1 and the data are summarized in Table 1. It is evident that oxygen does not enhance the radiation sensitivity, but in fact appears to reduce it. By contrast, the radiosensitivity under H_2 is clearly higher than that obtained with the deaerated (helium saturated) system.

Effect of N_2O (with and without O_2)

In order to study the effect of N_2O on radiosensitivity the enzyme solutions were bubbled (prior to irradiation) either with N_2O or with $N_2O + O_2$ (9:1 gas-mixture). The results are illustrated in Fig. 2 and the respective inactivation constants are summarized in Table 1. The replacement of helium by nitrous-oxide had reduced the radiosensitivity, whereas in the presence of both N_2O and O_2 an increase of the radiosensitivity, with a factor of about 1.3, was observed (a factor of 2 as compared with the radiosensitivity under N_2O alone).

Effect of radicals scavengers

The introduction of sodium formate (0.2M) to either deaerated or aerated enzyme solution in fact completely protected the enzyme from the radiodamage. The same result was obtained when the formate was replaced by polyethylene-glycol 5% W/V.

DISCUSSION

Enzyme inactivation

The full radioprotection afforded to the enzyme by the radical scavengers (formate or polyethylene glycol) shows that practically all of the radiodamage resulted from the attack of free radicals. With 0.6 μM enzyme, and in the absence of any other additives, the formed hydrated electrons react with the phosphate yielding H atoms:

Considering the rate constants of the relevant radical-radical and radical-enzyme reactions ($k_{\text{H}+\text{H}} = 6 \cdot 10^9 \text{ M}^{-1} \text{ sec}^{-1}$; $k_{\text{OH}+\text{OH}} = 7 \cdot 10^9 \text{ M}^{-1} \text{ sec}^{-1}$; $k_{\text{protein}+\text{H}} = 3 \cdot 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ (per residue); $k_{\text{protein}+\text{OH}} = 5 \cdot 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ (per residue) (17)), as well as the production rate of the radicals ($3 \cdot 10^{-8} \text{ M}^{-1} \text{ sec}^{-1}$ per G=1) and the enzyme concentration (0.6 μM , 237 residues per polypeptide), clearly demonstrates that practically all of the OH radicals and the majority of the H atoms react with the enzyme. Under such experimental conditions any contribution due to hydrated electrons, as was previously discussed (18), is actually eliminated as they are converted into H.

A comparison of the low value determined for G (0.055) with the known yield of the water-radicals ($G_{\text{OH}} + G_{\text{H}} + G_{e_{\text{aq}}^-} = 6.1$), shows that only a small fraction of them contributed to enzyme inactivation. The rest of the radicals reacted with the polypeptide chain without inducing any detectable enzyme inactivation. The observed exponential dependence of enzyme activity on dose is in accord with the assumption that the reaction rates of the water radicals with active and with inactive enzyme molecules do not differ, as was anticipated (16). This conclusion agrees with the assumption, made above, that most of the radicals react with the enzyme without an effect on enzyme activity.

The primary inactivating species

The conversion of the hydrated electrons into $\dot{\text{H}}$ atoms (via reaction 1) leaves $\dot{\text{H}}$ and $\dot{\text{OH}}$ as the sole potential damaging species. Oxygen, which efficiently reacts with $\dot{\text{H}}$ radicals and with e_{aq}^- , has provided the enzyme with partial radio-protection (see Fig. 1), thus suggesting that $\dot{\text{H}}$ atoms also contribute to the inactivation of the enzyme. The increase of radiosensitivity brought about by hydrogen is interpretable as follows: hydroxyl radicals react with the molecular hydrogen,

yielding $\dot{\text{H}}$ atoms. Consequently, the amount of $\dot{\text{H}}$ atoms is increased at the expense of the $\dot{\text{OH}}$. Thus the observed increase of the radiosensitivity, accompanying the introduction of H_2 , suggests that $\dot{\text{H}}$ atoms are more harmful than $\dot{\text{OH}}$ toward the enzyme.

Relative efficiencies of $\dot{\text{H}}$ and $\dot{\text{OH}}$

Evaluation of the relative contributions of $\dot{\text{H}}$ and $\dot{\text{OH}}$ to the radiodamage can be made considering the measured radiosensitivities of the enzyme under He, N_2O and H_2 . Assuming that each of the radical species acts independently of the other, with its own characteristic efficiency ((denoted $\langle \text{H} \rangle$ and $\langle \text{OH} \rangle$), and neglecting radical-radical reactions, the contribution to inactivation of each radical will be proportional to the product of the radical concentration and efficiency. Adopting 2.7, 2.7 and 0.7 as the respective values for G_{OH} , $G_{\text{e}_{\text{aq}}^-}$, and G_{H} and using the corresponding k values from Table 1, the following equations are obtained:

$$3.4 \langle \dot{\text{H}} \rangle + 2.7 \langle \dot{\text{OH}} \rangle = 0.095 \text{ krad}^{-1} \quad [3]$$

under He ($\text{e}_{\text{aq}}^- \rightarrow \dot{\text{H}}$)

$$0.7 \langle \dot{\text{H}} \rangle + 5.4 \langle \dot{\text{OH}} \rangle = 0.064 \text{ krad}^{-1} \quad [4]$$

under N_2O ($\text{e}_{\text{aq}}^- \rightarrow \dot{\text{OH}}$)

$$6.1 \langle \dot{\text{H}} \rangle = 0.12 \text{ krad}^{-1} \quad [5]$$

under H_2 (e_{aq}^- , $\dot{\text{OH}} \rightarrow \dot{\text{H}}$)

Here the notations $\langle \dot{\text{H}} \rangle$, $\langle \dot{\text{OH}} \rangle$ represent the respective "characteristic-inactivation-efficiency" of each of the two species (for $G = 1$). Equations 3-5 are valid, provided that all $\dot{\text{H}}$ atoms react with the enzyme rather than decay via self-recombination, and that H_2 successfully competes with the enzyme for the hydroxyl radicals. Solving equations 3 and 4 for $\langle \dot{\text{H}} \rangle$ and $\langle \dot{\text{OH}} \rangle$ yields the values of 0.0205 krad^{-1} and 0.009 krad^{-1} , respectively, a result which agrees with equation 5 as well. This result indicates that $\dot{\text{H}}$ atoms are more than twice as effective as $\dot{\text{OH}}$ radicals, inducing an enzyme inactivation. In fact, some recombination of $\dot{\text{H}}$ atoms occurs, and under H_2 part of the $\dot{\text{OH}}$ radicals react with the enzyme. Had we been able to correct equations 3-5 for those effects, we would have found that $\dot{\text{H}}$ is even more effective than previously found. When calculating the steady state concentration of the hydrogen atom for each case, equations [3] - [5] have been corrected for $\dot{\text{H}}$ self-recombination, and for the partial scavenging efficiency of $\dot{\text{OH}}$ by H_2 . The calculation showed that only about half of the $\dot{\text{OH}}$ radicals were converted by H_2 into $\dot{\text{H}}$ atoms, and that the fraction of $\dot{\text{H}}$ atoms reacting with the enzyme decreased with $[\text{H}]_{\text{s.s.}}$, being 90%, 69% and 64% under N_2O , He, and H_2 , respectively.

The modified equations thus obtained are:

$$3.4 \langle \dot{\text{H}} \rangle 0.69 + 2.7 \langle \dot{\text{OH}} \rangle = 0.095 \text{ krad}^{-1} \quad ['3]$$

$$0.7 \langle \dot{\text{H}} \rangle 0.9 + 5.4 \langle \dot{\text{OH}} \rangle = 0.064 \text{ krad}^{-1} \quad ['4]$$

$$4.85 \langle \dot{\text{H}} \rangle 0.64 + 2.7 \langle \dot{\text{OH}} \rangle 0.5 = 0.12 \text{ krad}^{-1} \quad ['5]$$

Evaluation of $\langle \dot{\text{H}} \rangle$ and $\langle \dot{\text{OH}} \rangle$ from equations ['3] and ['4] yielded 0.031 krad^{-1} and 0.0083 krad^{-1} , respectively, a result which reasonably agrees with equation ['5]. The damaging efficiency of H atoms appears to be almost four-fold higher than that of OH radical. Thus, in the presence of phosphate, it is H which is responsible for the majority of the radiodamage.

Role of superoxide radicals

The rate of enzyme inactivation under oxic conditions was, as seen in Fig. 1, lower than under helium. In the presence of oxygen, $\dot{\text{H}}$ and e_{aq}^- are replaced by $\dot{\text{O}}_2^-$. Hence, the character of the resulting oxygen effect (protective or sensitizing) would depend on the relative damaging efficiency of $\dot{\text{O}}_2^-$ as compared with $\dot{\text{H}}$ and e_{aq}^- . In order to decide whether $\dot{\text{O}}_2^-$ radicals are also involved in the radiodamage, the effect of formate in aerated system may be considered. There, practically all the water radicals are converted into superoxide radicals. Under these experimental conditions, actually no radiation-induced enzyme inactivation has been detected. In other words, the $\dot{\text{O}}_2^-$ radicals appeared to be completely inactive toward the enzyme. The result is anticipated, considering the very low reactivity of $\dot{\text{O}}_2^-$ and in light of the composition of the penicillinase whose polypeptide chain is devoided of SH groups (19).

Role of oxygen

Concluding that superoxide radicals are apparently inactive, implies that in the presence of oxygen, which converts e_{aq}^- and H into O_2^- , a protective effect might be anticipated. Indeed, such an effect is seen in Fig. 1. Yet, if the oxygen only eliminates the damage due to H atoms, the residual damage expected would be due to $\dot{O}H$ radicals (less than 25% of the total damage). The results shown in Fig. 1 prove that this is not the case, whereas in the presence of oxygen the damage was reduced to ~70% of its value under He. These results can be explained by assuming a double role for O_2 . On one hand, oxygen reduces the radiodamage by converting active damaging species (e_{aq}^- , H) into inactive ones (O_2^-), thus reducing the amount of the damaging primary hits. On the other hand, it increases the radiosensitivity by enhancing the damage already caused by $\dot{O}H$. If this is the case, the relative extents of these two opposing functions would determine the total net effect. In other words, either a sensitization or a protection or even a lack of effect might be observed. It seems that in the present system, the effect of protection exceeds that of sensitization.

To examine the assumption of a double role for O_2 , the effect of oxygen in a system where e_{aq}^- and H were not replaced by inactive O_2^- radicals was considered.

Combined effect of O_2 and N_2O

In solutions saturated with $N_2O + O_2$ gas mixture, the concentrations ratio of the two gases $[N_2O]/[O_2]$ is about 150 (due to the higher solubility of N_2O in aqueous media). Taking into account the bimolecular reaction rate constants of N_2O and O_2 with e_{aq}^- (17), it is evident that N_2O competes more successfully for the

hydrated electrons than does the oxygen or the phosphate. Consequently, the majority of the e_{aq}^- would react with N_2O giving rise to $\dot{OH}$, rather than with oxygen to yield $\dot{O}_2^-$. This makes it possible to study the oxygen effect while minimizing the formation of the $\dot{O}_2^-$. Examination of Fig. 2 indicates that with $N_2O + O_2$, a considerable radiosensitization is achieved. This is in agreement with the assumption made above that the oxygen is involved in two different independent effects.

In the presence of oxygen the only damaging species are the hydroxyl radical, as $\dot{H}$ and e_{aq}^- are converted into harmless $\dot{O}_2^-$. Therefore taking into account the oxygen enhancement ratio (OER) and using the corresponding inactivation constants from Table I the following equations are obtained:

$$2.7 \quad \langle OH \rangle \quad OER = 0.062 \text{ krad}^{-1} \quad [6]$$

under oxygen (e_{aq}^- , $\dot{H}$, $\rightarrow \dot{O}_2^-$)

and

$$5.4 \quad \langle OH \rangle \quad OER = 0.125 \quad [7]$$

under $N_2O + O_2$ ($\dot{H} \rightarrow \dot{O}_2^-$, $e_{aq}^- \rightarrow \dot{OH}$)

Taking $\langle OH \rangle = 0.0083 \text{ krad}^{-1}$ (for $G=1$), as obtained above, and solving any of these equation yields the value of 2.8 for OER. In case that the value of $\langle OH \rangle = 0.009 \text{ krad}^{-1}$ would have been taken (evaluated using equations [3] - [5]), then the OER obtained would amount to 2.6 only).

The present results indeed suggest that molecular oxygen enhances the radiodamage of $\dot{OH}$ almost three fold, but with penicillinase this effect is masked by the protective effect. Since repair mechanisms do not operate within isolated enzymic systems, it is very likely that the sensitizing effect of oxygen involves the prevention or

reduction of chemical restitution processes. In buffered solutions of the enzyme devoid of any additives, such processes might take place either inter- or intramolecularly.

CONCLUSIONS

- 1) Both $\dot{\text{H}}$ and $\dot{\text{OH}}$ inactivate the enzyme.
- 2) The efficiency of $\dot{\text{H}}$ exceeds that of $\dot{\text{OH}}$ by about four fold.
- 3) $\dot{\text{O}}_2^-$ does not inactivate the enzyme.
- 4) Oxygen plays a double role in this system; it protects by scavenging $\dot{\text{H}}$ atoms and e_{aq}^- , and enhances the damage caused by $\dot{\text{OH}}$. In this system some protection by O_2 is observed as the former effect exceeds the latter.

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TABLE 1

Inactivation constants for penicillinase γ -irradiated under H_2 , O_2 , N_2O and He^(a)

Saturating Gas	Inactivation Constant k (krad^{-1})
He	0.095
O_2	0.062
N_2O	0.064
N_2O+O_2 ^(b)	0.125
H_2	0.120

(a) 0.6 μ M enzyme dissolved in 50mM phosphate buffer (pH 7).

(b) 9:1 gas-mixture of N_2O + O_2 .

LEGENDS TO FIGURES

Fig. 1. Enzymatic activity of penicillinase vs. dose, after γ -irradiation at $1.7 \text{ krad} \cdot \text{min}^{-1}$, of $0.6 \mu\text{M}$ enzyme in 50mM phosphate buffer pH 7, at room temperature. The enzyme solutions were saturated with:
(o) helium; ($\square$) oxygen; (Δ) hydrogen.

Fig. 2. Effect of N_2O with and without oxygen on the radiation-induced enzyme inactivation. Enzymatic activity of penicillinase vs. dose. Experimental conditions as in Fig. 1.
($\square$) N_2O saturated; (o) saturated with $\text{N}_2\text{O} + \text{O}_2$, 9:1 gas mixture.