On the X-Ray Inactivation at Various Temperatures of Trypsin in Dry State and Aqueous Solution

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The X-ray sensitivity of trypsin has been studied both in dry state and in dilute solution (0.1 mg/ml) of 10^{-3} N HCl as a function of the sample temperature during irradiation. Oxygen present during irradiation acts as a sensitizer when the exposure is carried out in frozen aqueous solution as well as in dry state, but it acts as a protector when the enzyme solution is exposed at room temperature. There is a considerable indirect effect when trypsin is exposed in frozen aqueous solution, above 200 °K, and even at 77 °K a small indirect effect is apparent.

When trypsin is exposed in 10^{-3} N HCl at room temperature about 85 per cent of the indirect effect is eliminated when the solution is saturated with O_{2} (known as an effective scavenger for e^{\circ} and H) and most of the remaining indirect effect is eliminated if the enzyme solution also contains 100 mM C_{2}H_{5}OH (known as an effective OH scavenger).

It is known that a dose of the order of a few krads may be sufficient to destroy the activity of enzymes in aqueous solutions, whereas the same dose given to enzymes in the dry state or in frozen aqueous solutions has only little effect.

The so-called “direct” effect on enzymes is generally assumed to result from a primary ionization event within the confines of the enzyme or its immediate vicinity. The additional radiation effect on enzymes irradiated in dilute aqueous solution is often referred to as the “indirect” effect, and assumed to be due to radiolysis products of water, notably free radicals and other toxic products.

The concepts “direct” and “indirect” effects are, however, quite ambiguous. Thus, numerous agents are known to affect the degree of radiation injury of dried macromolecules and energy transfer processes, as seen by ESR technics, occur in dried macromolecules as well as in frozen aqueous solutions.

Among the primary radiolysis products of water there are reasons to suspect hydroxyl radicals (OH), atomic hydrogen (H) and hydrated electrons (e^{\circ}) to be particularly important for the X-ray induced inactivation of enzymes in dilute aqueous solution. Furthermore, it is established that O_{2} reacts readily with H and e^{\circ}, and that C_{2}H_{5}OH is an effective scavenger of OH radicals. Thus, it may be possible to reduce the combined effect of H and e^{\circ} by flushing O_{2} through the aqueous solution during irradiation, and to suppress the effect of OH by means of ethanol.

The purpose of the present paper was to determine the radiosensitivity for inactivation of trypsin in dry state and in aqueous solution as a function of the sample temperature during X-ray irradiation, and by means of radical scavengers to throw some light on the relative contribution to the enzyme inactivation in aqueous solution at room temperature, from oxidizing and reducing radiolysis products.

Materials and Methods

Enzyme

The enzyme used in the present investigation was twice-crystallized, salt-free trypsin from Worthington Biochemical Corporation. The enzyme was dissolved in 10^{-3} N HCl to a concentration of 0.1 mg/ml. The trypsin solutions were freshly prepared prior to each experiment.

Radiation Sources

Two different radiation sources were used. For determination of the radiosensitivity of trypsin as a function of the sample temperature, 45 kV unfiltered X-rays from a Machlett tube with beryllium window were used. All the other experiments reported here were carried out with X-rays from a Siemens Stabilipan X-ray unit. The irradiation was carried out using a tube voltage of 220 kV, 0.5 mm Cu-filter, and a dose rate of 1.2 krad/min.

Preparation of Samples

Samples of enzymes in aqueous solution were prepared for exposure to 45 kV X-rays by pipetting aliquots of 50 µl of the enzyme solution onto small bombardment vessels or disks of glass. Dried samples of trypsin were prepared by placing disks so prepared in a desiccator and applying a vacuum of about \(10^{-3}\) mm Hg for about 14 hours. The desiccator was opened in the same atmosphere as used during the subsequent irradiation.

In order to irradiate samples at various constant temperatures the disks were placed on top of a copper rod partly immersed in a Dewar bottle containing a desired cooling medium, as shown in Fig. 1. Nitrogen and oxygen are lead through a cooling trap when trypsin is irradiated in the dry state and in frozen aqueous solution, and through a gas washing bottle when samples are irradiated in the liquid state. Samples exposed to 220 kV X-rays were prepared by adding 10 ml of the enzyme solution to an irradiation vessel of the type used by Howard-Flanders and Alfer. This chamber allowed continuous gas bubbling of the enzyme solution prior to and during irradiation and aliquots could be extracted without admitting atmospheric air or interrupting the gas flow. The gases used in the present investigation were \(N_2\) and \(O_2\), each with at least 99.99 per cent purity, and with less than 0.005 per cent \(O_2\) in the \(N_2\), as specified by the manufacturer. These gases are referred to as “100 per cent \(N_2\)”, respectively “100 per cent \(O_2\)”.

Preparation of Gas Mixtures

In order to determine the radiosensitivity as a function of the concentration of dissolved \(O_2\) in the enzyme solution, a pressure tank was used to mix \(O_2\) and \(N_2\) to known partial pressures. The enzyme solution was bubbled with such gas mixtures prior to and during the exposure, and the concentration of \(O_2\) at equilibrium condition was determined according to Henry’s law, taking into consideration the solubility of the gases in water at appropriate temperature and partial pressure.

Dosimetry

Determination of dose rates was carried out both with the ferrous sulphate dosimeter and with a Victoren type ionization chamber. The results agreed to within 10 per cent. The doses given in the present paper are all based on the ferrous sulphate dosimeter.

Under otherwise equal irradiation conditions, the dose rates were assumed equal in dried samples and in aqueous samples, because the absorption coefficients of crystallized trypsin and water do not differ significantly for photonenergies between 10 keV and 50 keV.

Determination of Remaining Enzymatic Activity

The enzymatic activity was determined according to the method described by Schwert and Takekaka. Benzoyl-L-arginine ethyl ester (BAEE) obtained from Worthington Biochemical Corporation, was used as substrate, dissolved in 0.05 M “Sørensen” phosphate buffer pH 8 to a concentration of 7.7 \(10^{-2}\) mg/ml. Samples (50 µl) of the trypsin solution were taken before and at intervals during the period of irradiation. Each sample was mixed with 1 ml of \(10^{-3}\) N HCl and 2 ml substrate solution, and the optical density of the mixture was recorded as a function of time, by means of a Zeiss spectrophotometer, at 2530 Å. The remaining enzymatic activities of irradiated samples, derived from the slopes of such curves, were expressed as percentages of the activity of the unirradiated controls.

12 S. C. Dhar and S. M. Bose, C. A. 56, 5088 a [1962].

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Fig. 1. Experimental setup when trypsin is irradiated with 45 kV X-rays. Constant sample temperature were obtained by using different cooling media: liquid \(O_2\) at 90 °K, \(CO_2\)-ice and \(C_2H_5OH\) (96%) at 200 ± 2 °K, \(CO_2\)-ice and water at 263 °K and ice and water at 273 °K.
Results

Dose-effect Curves

Exponential dose-effect curves were found for dry trypsin (Fig. 2), for trypsin in frozen aqueous solution (Fig. 3), and for trypsin exposed to 45 kV X-rays in non-frozen aqueous solution (Fig. 4). Two types of non-exponential curves were observed after exposure of the aqueous enzyme solution to 220 kV X-rays, namely:

1. Curves with an initial low sensitivity for inactivation, followed by an approximately exponential portion with higher radiosensitivity (Fig. 7).

2. Curves similar to those described above, but without any clear exponential region. Such curves were always found when C₂H₅OH was present in the solution during irradiation (Fig. 9). We shall return to a possible explanation for this latter curve shape.

There is no general rule as to how to define radiosensitivity when the dose-effect curves differ in shape as was the case in the present investigation. In this

Fig. 2. Dose-effect curves obtained after exposure at room temperature and in 100 per cent N₂-atmosphere.

Fig. 3. Dose-effect curves obtained after exposure at 263 °K of trypsin in frozen aqueous solution to 45 kV X-rays in 100 per cent O₂-atmosphere and 100 per cent N₂-atmosphere.

Fig. 4. Dose-effect curves obtained after exposure at room temperature of trypsin in aqueous solution to 45 kV X-rays in 100 per cent O₂-atmosphere and 100 per cent N₂-atmosphere. The dose rate was varied from 1.2 krad/min to 103 krad/min.

Fig. 5. O₂-enhancement ratio as a function of the irradiation temperature for trypsin in aqueous solution and in dry state; 45 kV X-rays.

Fig. 6. Radiosensitivity as a function of the irradiation temperature. Trypsin samples exposed in dry state and in aqueous solutions to 45 kV X-rays in 100 per cent O₂-atmosphere and in 100 per cent N₂-atmosphere.
paper the radiosensitivity is expressed in two different ways, namely as:

1. the reciprocal value of the dose which reduces the enzymatic activity to \(1/e\) or 37\% of that of the unirradiated control, \(1/D_{37}\) (rad\(^{-1}\)),

2. the slope of the tangent to the dose-effect curve in the low dose region, \(\alpha\), derived from the expression \(f = e^{-\alpha D}\), where \(f\) is the fraction of the enzymatic activity remaining after a dose \(D\) (rad) is delivered to the sample.

Effect of Temperature on Radiosensitivity and \(O_2\)-enhancement Ratios

The dose-effect curves obtained after exposure of dried trypsin in atmospheres of 100 per cent \(O_2\) and 100 per cent \(N_2\) are given in Fig. 2 and show that \(O_2\) acts as a sensitizer relative to \(N_2\) when the injury results predominantly from direct effect. As shown in Fig. 3 similar results were obtained when trypsin was irradiated in frozen aqueous solutions. The present finding that the radiosensitivity of dried trypsin is greater in \(O_2\)- than in \(N_2\)-atmosphere is in accordance with results obtained by others\(^{14,15}\), whereas the observation that the \(O_2\)-effect of trypsin is greater than one in frozen solution is new.

Fig. 4 shows dose-effect curves obtained for trypsin in aqueous solution exposed at room temperature. Contrary to the results obtained for dried trypsin and for trypsin in frozen aqueous solution, \(O_2\) acts here as a radiation protector compared to \(N_2\). It follows from Fig. 4 that a dose-rate variation from 1.2 krad/min to 103 krad/min neither affects the radiosensitivity nor the oxygen enhancement ratio, \(\alpha\), e.g. the ratio between the radiosensitivity in \(O_2\) and that in \(N_2\).

A summary of the \(O_2\)-enhancement ratios observed for trypsin irradiated in dry state and in aqueous solution at different temperatures, is shown in Fig. 5. As seen, the \(O_2\)-enhancement ratio is almost constant for trypsin in dry state and \(\alpha \approx 1.5\). Also for trypsin irradiated in frozen aqueous solution is \(\alpha > 1\). The \(O_2\)-enhancement ratio under the latter conditions, however, decreases slowly for increasing temperatures. Around the melting point of the enzyme solution an abrupt drop to a value less than one is observed. Thus, depending on the

\(^{14}\) P. Alexander, Radiat. Res. 6, 653 [1957].

sample temperature during irradiation the oxygen enhancement ratio of trypsin in dilute solution may be $\geq 1$.

Fig. 6 shows the radiosensitivity of trypsin in dry state and in aqueous solution, as a function of the sample temperature during irradiation. As seen from the figure, the radiosensitivity of dried trypsin and of trypsin in solution increases with increasing temperature. However, the radiosensitivity of trypsin in solution is greater than that in dry state at all temperatures investigated. Furthermore, in frozen aqueous solution trypsin does show a greater increase in radiosensitivity with temperatures exceeding 200 $^\circ$K than in dry state. When the sample temperature is increased from 263 $^\circ$K (frozen sample) to 273 $^\circ$K (sample in liquid state) the radiosensitivity in N$_2$-atmosphere increases by more than two orders of magnitude. It has been shown that also for lysozyme in dilute aqueous solution the radiosensitivity is much higher above than below the freezing point$^1$. Above 273 $^\circ$K the sensitivity again rises less abruptly with increasing temperature.

Fig. 7 shows dose-effect curves obtained after exposure of trypsin in aqueous solution containing different concentrations of O$_2$, and Fig. 8 shows the radiosensitivity of trypsin derived from the curves shown in Fig. 7 as a function of the molar concentrations of O$_2$ in the solution. As seen, the radiosensitivity decreases considerably for small O$_2$ concentrations, approaching a saturation level as the O$_2$ concentration is increased. With an O$_2$ concentration of 5 $\mu$M the radiosensitivity was found to be about 50 per cent less than that obtained in the absence of oxygen.

**Effect of C$_2$H$_5$OH**

Fig. 9 shows the dose-effect curves obtained after exposures of trypsin in O$_2$-saturated aqueous solution in the presence of different concentrations of C$_2$H$_5$OH. The dose-effect curve for dried trypsin flushed with O$_2$ is indicated on this figure. Fig. 10 shows the radiosensitivity of trypsin in aqueous solution as a function of the molar concentration of C$_2$H$_5$OH. As seen from the figures the radiation protection increases with increasing C$_2$H$_5$OH concentration approaching a saturation value.

For ethanol concentrations exceeding 0.1 mM it can easily be seen that the dose-effect curves do not exhibit any exponential portion, in contrast to those obtained under all other conditions. At the highest ethanol concentration (100 mM) the slope of the dose-effect curve in the low dose region appears to be close to that for dried trypsin. However, as the dose increases the rate of inactivation increases when ethanol is present and the dose-effect curve falls below that for dried trypsin.

This anomalous shape of the dose-effect curves in the presence of ethanol led us to suspect the ethanol of being degraded by the radiation in such a way that its protective power hereby becomes lost. In order to test this assumption a 1 mM ethanol solution, less enzyme, was irradiated under oxygen bubbling, and given doses of 148 krad, 222 krad and 296 krad. Trypsin was then dissolved (0.1 mg/ml) in these pre-irradiated solutions, and dose-effect curves determined in subsequent irradiation experiments. Fig. 11 shows the results of these tests. The
protective effect of oxygen saturated ethanol is reduced when the solution is pre-irradiated, and more so the higher the dose. Pre-irradiation with a dose of about 0.3 M rad appears to abolish the protective effect more or less completely. From these data one can conclude that C$_2$H$_5$OH is degraded during irradiation, and this tends to reduce the scavenging effect progressively, thus giving rise to dose-effect curves of the shape shown in Figs. 10 and 11. The use of 1/D$_{37}$ is therefore not justified as a measure of radiosensitivity under these conditions. Instead it appears more appropriate to use the negative slope of the tangent to the dose-effect curves in the low dose region.

**Discussion**

The data presented in Fig. 5 show that oxygen enhances the radiosensitivity under two conditions, namely in dry state and in frozen solutions. Such enhancement of the radiosensitivity by oxygen is analogous to that characteristic for cellular systems, and may be caused by a mechanism such as that suggested by Howard-Flanders 16.

From Fig. 6 it is seen that the radiosensitivity of trypsin in frozen aqueous solution always is somewhat greater than that in dry state at a given temperature, and more so the higher the temperature. This finding is in agreement with the ESR results obtained by Sanner 17 which indicate that in frozen solutions some indirect effect may occur even at 77 °K. At this temperature the indirect effect was found largely to be due to the action of water radicals formed in a thin shell of bound water surrounding each solute molecule. With increasing irradiation temperature Sanner found a greater contribution to the indirect effect from diffusible water radicals, formed outside this shell.

In contrast to the protective effect of oxygen referred to above, it is seen from Fig. 5 that oxygen reduces the radiosensitivity as compared to nitrogen when the enzyme solution is irradiated at room temperature.

From Fig. 6 it is seen that the radiosensitivity under anoxia is about 2000 times greater when trypsin is irradiated in aqueous solution as compared to that for dried enzymes. Furthermore, from Figs. 9 and 10 it is seen that ethanol present during irradiation suppresses the degree of this indirect effect considerably.

In the following attempts will be made to explain the observed reduction of the indirect effect by oxygen and ethanol on the basis of known properties of these compounds to act as radical scavengers. The contribution to the indirect effect from different aqueous radiation products may be derived on the assumption that O$_2$ scavenges H and e$_{aq}^-$, and C$_2$H$_5$OH scavenges OH radicals 7-8.

The following analysis is to be considered only as a rough approximation, because the water radicals do not necessarily act independently, and scavenging of one species may affect the concentration of other types of species which are left unaffected by a particular scavenger.

Table I shows the rate constants ($k$) and reaction rates ($kC$) for reactions of e$_{aq}^-$, H and OH with H$^\circ$, O$_2$, C$_2$H$_5$OH and trypsin at pH about 3. As seen from the table the reaction rate of H$^\circ$ with e$_{aq}^-$ at this pH is quite similar to that of O$_2$ with e$_{aq}^-$ or O$_2$ with H. Almost all radiation induced e$_{aq}^-$ will appear

<table>
<thead>
<tr>
<th>Rate constants, $k$ (M$^{-1}$ sec$^{-1}$)</th>
<th>Reaction rates, $kC$ (sec$^{-1}$)</th>
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<tbody>
<tr>
<td>H$^\circ$ 2 · 10$^{10}$ O$_2$ 2 · 10$^{10}$ C$_2$H$_5$OH 1.7 · 10$^7$ Trypsin* 10$^{11}$</td>
<td>H$^\circ$ 200 · 10$^5$ O$_2$ 250 · 10$^5$ C$_2$H$_5$OH 10$^4$ Trypsin (4 · 10$^{-6}$ M)</td>
</tr>
<tr>
<td>H small 2 · 10$^{10}$ OH small 2 · 10$^{10}$</td>
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Table I 8,18,19. Rate constants ($k$) and reaction rates ($kC$) for reactions of e$_{aq}^-$, H and OH with H$^\circ$, O$_2$, C$_2$H$_5$OH and trypsin in acid solution (pH about 3). * No exact values of these rate constants are at present available. However, according to the method suggested by Braams 16,18, based on the rate constants of e$_{aq}^-$ with the different amino acids, the constants are all less than 10$^{11}$ M$^{-1}$ sec$^{-1}$.

16 P. Howard-Flanders, Advances biol. med. Physics 6, 553 [1958].
as H atoms under irradiation of $10^{-3}$ N HCl in N$_2$-atmosphere. Thus, among the primary radiolysis products H and OH may be suspected of being of particular importance for the radiation injury in this system. With O$_2$ in the solution during irradiation the reaction can be written as suggested by Schooles.  

$$H + H_2 \rightarrow HO_2.$$  

$$e^- + O_2 \rightarrow O_2^\circ \rightarrow H + O_2^\circ \rightarrow H^+ + O_2^\circ \ (pK \sim 4).$$

With 1.25 mM O$_2$ in the solution the $kC$ product (Table I) for the reaction between H and O$_2$ is at least 60 times greater than that between H and trypsin. This indicates that O$_2$ is present in such an abundance that most of the H atoms are expected to react with oxygen instead of with trypsin.

<table>
<thead>
<tr>
<th>Exposure conditions</th>
<th>Radiosensitivity, $\text{rad}^{-1}$</th>
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<tbody>
<tr>
<td>Solution, 100% N$_2$</td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td>Solution, 1.25 mM O$_2$</td>
<td>$1.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Solution, 1.25 mM O$_2$ + 100 mM C$_2$H$_5$OH</td>
<td>$9.0 \times 10^{-7}$</td>
</tr>
<tr>
<td>Dried trypsin, 100% O$_2$</td>
<td>$6.3 \times 10^{-8}$ *</td>
</tr>
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Table II. Radiosensitivities of trypsin under four different experimental conditions. The sensitivities are calculated from the slope of the tangent to the dose-effect curve in the low dose region, $-\alpha$. From these sensitivities it seems (see text) as if about 85% of the radiation injury under anoxia is due to H($+e^-O_2^\circ$), whereas most of the remaining injury may be caused by OH radicals. * Butler and Robins report 11.4 $\times 10^{-6}$. Hutchinson and Watts report 4.0 $\times 10^{-6}$.

Since it is known that hydroperoxyradicals do not readily react with organic solutes, it seems reasonable to assume that the difference between the radiation injury observed in N$_2$-atmosphere and that in O$_2$-atmosphere reflects action of H-atoms. From the data presented in Table II it follows that on the above assumption about 85 per cent of the observed indirect effect is due to H-atoms.

With C$_2$H$_5$OH in the solution during irradiation the reaction can be written as suggested by Baxendale:

$$C_2H_5OH + OH \rightarrow C_2H_4OH + H_2O.$$  

$$C_2H_4OH + O_2 \rightarrow C_2H_2O + H_2O.$$  

With 100 mM C$_2$H$_5$OH in the solution the $kC$ product (Table I) for the reaction between OH and C$_2$H$_5$OH is about 500 times greater than that between OH and trypsin. This indicates that in a 100 mM ethanol solution most of the OH radicals will react with ethanol instead of with trypsin. Thus, to a first approximation one may suggest that the difference between the injury in O$_2$-atmosphere and that in oxygen saturated 100 mM ethanol solution remaining indirect effect is due to OH-radicals.

Thus, from a simple first approximation analysis it appears from Table II that probably 85 per cent of the injury is due to H-atoms and most of the remaining indirect effect is due to OH-radicals.