GAMMA-RAY INACTIVATION OF TRYPSIN IN SOLUTION: EFFECTS OF VARIOUS SCAVENGERS

by B. B. SINGH, *Biology Division, Bhabha Atomic Research Centre, Trombay, Bombay, India, and* A. KABI, *Physics Department, Royal Military College of Science, Shrivenham, Swindon, Wiltshire, England*

(Communicated by A. R. Gopal-Ayengar, F.N.I.)

(*Received 12 July 1968; after revision 16 September 1968*)

The mechanism of inactivation of trypsin by ionizing radiations in aqueous solutions has been investigated using scavengers for various radiolytic products of water. It has been shown that OH radical scavengers, CNS^- and I^- offer better protection than alcohols and this difference could be explained on the basis of the reaction of the enzyme molecules with the radicals formed on alcohols. Electron scavengers also offered protection but to a lesser extent. Oxygen was found to be a better protector than NO_3^-. Iodoacetamide (ICH_2CONH_2) was shown to be by far the best protector because it scavenged e_{aq}^- forming I^- which subsequently scavenged the hydroxyl radicals. It has been concluded that major species responsible for the inactivation are hydroxyl radicals although e_{aq}^- cannot be altogether neglected.

INTRODUCTION

In the earlier studies on the inactivation of enzymes by ionizing radiation it has been suggested that intramolecular transfer of energy plays a prominent role (Augenstein and Grist 1962) although recent investigations have shown that the observed effects can be explained in terms of reaction of the enzyme molecules with the various intermediate species produced during radiolysis of water (Robins and Butler 1962; Brustad 1966).

The main products formed in radiolysis of water are: e_{aq}^-, H, OH, H_2 and H_2O_2. Although the radiation-induced inactivation of enzymes may be due to various processes mediated by these radiolytic products of water, it has not yet been possible to specify any particular species to be responsible for the inactivation process. It is, however, generally accepted that OH radicals are the main species contributing to the inactivation process. The effect of H atoms and e_{aq}^-, on the other hand, is more obscure although recently it has been shown that H atoms produced in an electrodeless discharge tube are capable of inactivating trypsin and chymotrypsin (Mee *et al.* 1964, 1965).

In the present investigation the radiation-induced inactivation of trypsin dissolved in phosphate buffer has been studied. From competitive kinetic studies using various radical scavengers for the primary radiolytic products of

water, e.g. H, OH, e^-_{aq}, we have attempted to specify the contributions of various species to the inactivation of the enzyme in solution.

Materials and Methods

Salt-free trypsin (6 × crystallized) and its substrate α-benzoyl, L-arginine, ethyl/ester, HCl (BAEE) were purchased commercially. All other chemicals were of analytical reagent grade.

Solutions of the enzyme were prepared in phosphate buffer (0·025 M KH_2PO_4+0·025 M $NaHPO_4$ in triple-distilled water). 5 ml of the enzyme samples containing the scavengers were irradiated at 0 °C with Co^{60} gamma-rays at a dose rate of 293 Krads/hr determined by Fricke-dosimeter assuming $G_{Fe^{+++}} = 15{\cdot}5$.

The enzymatic activity was determined by the method discussed by Schwert *et al.* (1948).

Results and Discussion

Although the loss of enzymatic activity due to ionizing radiations does not always bear an exponential relation with the absorbed dose (Anbar and Levitzki 1966) such a relation was found to hold true for trypsin in the present series of investigations. Figures 1 and 2 show the percentage residual activity of the enzyme as a function of dose. It is evident from these that the presence of various additives in the enzyme solution did not affect the exponential nature of the relationship between the inactivation and the absorbed dose. The D_{37} value (i.e. the dose required to reduce the enzymatic activity to 37 per cent of its initial value) has, therefore, been used as the criterion for the extent of radiation-induced inactivation.

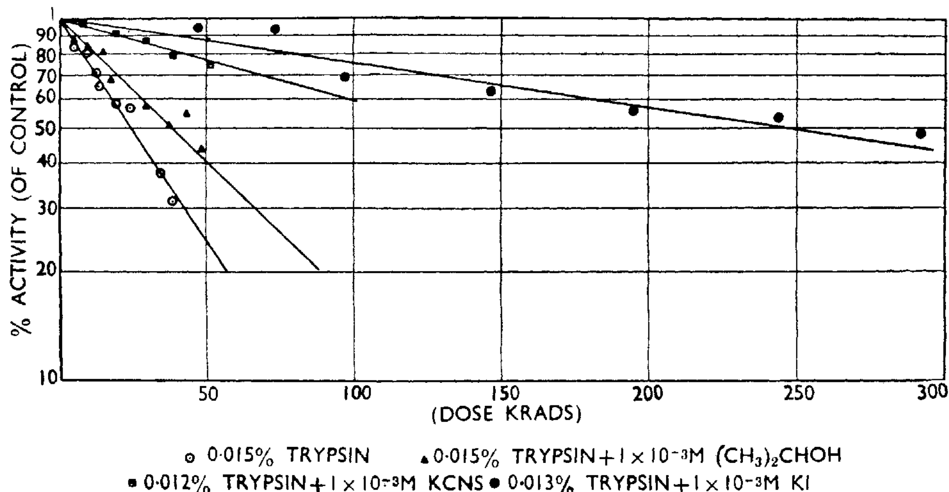


Fig. 1

The various additives investigated in the present system can be divided into three categories. Two of these groups are specific for scavenging hydroxyl radicals and solvated electrons respectively, while the third group consists of compounds capable of scavenging both e_{aq}^- and OH radicals. The D_{37}

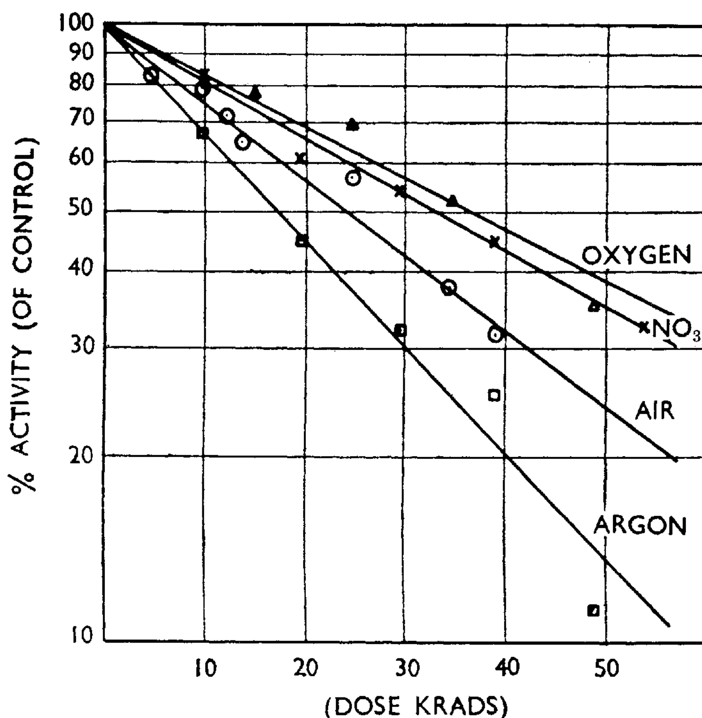


FIG. 2

values for the enzyme in the presence of various additives are shown in Table I.

Hydroxyl Radical Scavengers

Alcohols have generally been used for the scavenging of hydroxyl radicals due to their high reactivities towards these species (Anbar and Neta 1965). The results shown in Table I clearly indicate that alcohols do offer a certain amount of protection to the enzyme and this could be attributed, in part, to the scavenging of the hydroxyl radicals produced in the aqueous medium. However, the degree of protection obtained is rather small in spite of the fact that other investigators have presented indirect evidence suggesting hydroxyl radicals to be the major inactivating species for trypsin. In Table I the results on two other hydroxyl radical scavengers, namely CNS^- and I^-, are also shown. It can be seen that these two scavengers offered a better

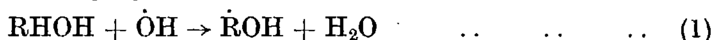
TABLE I

Conc. of trypsin (%)	Scavenger	Conc. of scavenger (M)	D_{37} (Krads)	G. values
0·015	–	–	35·0	0·178
	(a) *OH radical scavengers*			
0·014	Methanol	1×10^{-4}	37·2	0·156
0·015	Ethanol	1×10^{-4}	41·0	0·152
0·017	2-Propanol	1×10^{-4}	55·5	0·127
0·014	Propane-1, 3-diol	1×10^{-4}	55·5	0·105
0·017	Butane-1, 4-diol	1×10^{-4}	62·0	0·114
0·012	Pentane-1, 5-diol	1×10^{-4}	43·3	0·115
0·016	Glycerol	1×10^{-4}	39·9	0·166
0·014	KCNS	1×10^{-3}	180·0	0·032
0·013	KI	1×10^{-3}	370·0	0·014
	(b) e^-_{aq} *scavenger*			
0·013	O_2	$1{\cdot}4\times10^{-3}$	52·5	0·103
0·013	NO_3	1×10^{-3}	47·5	0·114
0.016	(c) e^-_{aq} *and OH radical scavenger*			
0·016	ICH_2CONH_2	1×10^{-3}	391·0	0·017

G. value = Number of molecules destroyed per 100 eV of absorbed energy.

protection than the alcohols, although the reactivities of alcohols, CNS^- and I^-, towards hydroxyl radicals are comparable (Anbar and Neta 1965).

The reaction of hydroxyl radicals with the above scavengers can be represented by the following equations:



$$RHOH + \dot{O}H \rightarrow \dot{R}OH + H_2O \quad \ldots \quad (1)$$

$$X^- + \dot{O}H \rightarrow \dot{X} + OH^- \quad \ldots \quad (2)$$

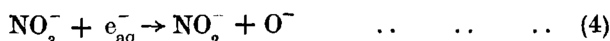
where RHOH = alcohol and $X^- = CNS^-$ or I^-. It is evident from equation (1) that the reaction of OH radicals with alcohols leads to the production of alcohol radicals which generally decay forming aldehydes by disproportionation. The subsequent reactions of the radicals $CN\dot{S}$ and $\dot{I}$ have also been investigated and in general these species are found to dimerize giving $(CNS)_2$ and I_2 respectively. It should, however, be pointed out that dimerization is comparatively a more favourable phenomenon than disproportionation of radicals and taking this fact into consideration the relatively small protection shown in the case of alcohols can be explained in terms of reaction of $\dot{R}OH$ radicals with the enzyme molecules leading to the inactivation of the latter. Although this is a likely hypothesis, there is at present no experimental evidence to confirm such a mechanism.

Furthermore, it can also be seen from Table I that diols were in general (except pentane 1–5 diol) a better class of protectors than the monohydric or polyhydric alcohols. A similar phenomenon has been reported in bacterial systems which was explained in terms of the hydrophobic characteristics of the alcohols (Siddle 1964). Such a mechanism to explain the present results is rather unlikely.

Solvated Electron Scavengers

Figure 2 shows the effect of two electron scavengers and it is clear that oxygen offers a better protection than NO_3^-. The reaction of solvated electrons with O_2 or NO_3^- leads to the formation of comparatively unreactive species (Gordon *et al.* 1965; Hyder 1965), i.e.

$$O_2 + e_{aq}^- \rightarrow O_2^- \quad \ldots \quad (3)$$



$$NO_3^- + e_{aq}^- \rightarrow NO_2^- + O^- \quad \ldots \quad (4)$$

Thus, if e_{aq}^- are the species contributing to the inactivation process, both O_2 and NO_3^- would show a protective effect since O_2^- and NO_2^- are less reactive than e_{aq}^-. The experimental results obtained using O_2 as a scavenger are, however, rather difficult to interpret. In radiation biology, oxygen is a well-known radiosensitizer and this has been explained in terms of formation of peroxy radicals on the macromolecules. In isolated enzyme systems the results obtained so far are not quite clear. Many cases are known where oxygen acts as a protector and this protection ability is shown to have a strong dependence on environmental conditions such as *p*H of the enzyme solution (Robins and Butler 1962; Sanner 1965; Brustad 1966).

From a graphical plot of 1/G (inactivation) *vs.* (O_2) it has been found that $k_{e^-aq+Enz} = 15\times10^{10}M^{-1}\ sec^{-1}$. The experimental values obtained using the pulse radiolysis (Braams 1967) technique are comparatively smaller than this which indicates that e_{aq}^- are not the only species causing the inactivation although their contribution to the inactivation process cannot be neglected. It has been reported earlier that iodoacetamide (ICH_2CONH_2) reacts with solvated electrons forming iodide ions which subsequently react with the hydroxyl radicals producing iodine (Singh *et al.* 1966).

$$ICH_2CONH_2 + e_{aq}^- \rightarrow I^- + \dot{C}H_2CONH_2 \quad \ldots \quad (5)$$

$$I^- + OH \rightarrow I + OH^- \quad \ldots \quad (6)$$

$$I + I^- \rightarrow I_2^- \quad \ldots \quad (7)$$

$$2I_2^- \rightarrow I_3^- + I^- \quad \ldots \quad (8)$$

Direct evidence for these reactions is presented in Fig. 3 which shows the transient absorption spectrum during the pulse radiolysis of argon and nitrous

oxide saturated aqueous solutions of iodoacetamide. It shows two peaks at 390 nm and 240–260 nm which were found to decay by second order kinetics and can be tentatively assigned to I_2^- and $\dot{C}H_2CONH_2$ radicals respectively.

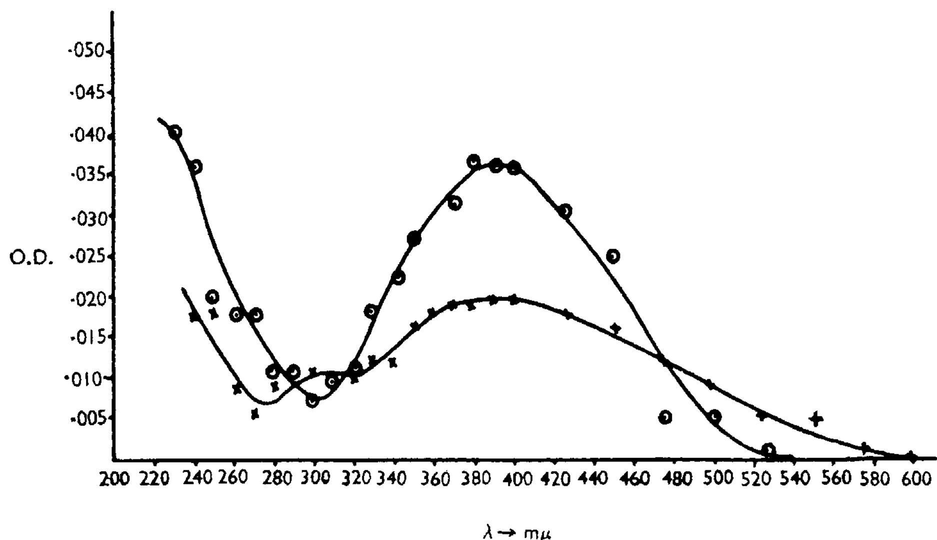


FIG. 3

Considering the reactions (5) and (6) it can be postulated that iodoacetamide would be a good radioprotector since K_5 and K_6 are estimated to be very high ($K_5 = 5 \times 10^{10} M^{-1}$ sec^{-1} and $K_6 = 1{\cdot}1 \times 10^{10} M^{-1}$ sec^{-1}). In fact, it can be seen from Fig. 4 and Table I that iodoacetamide is by far a

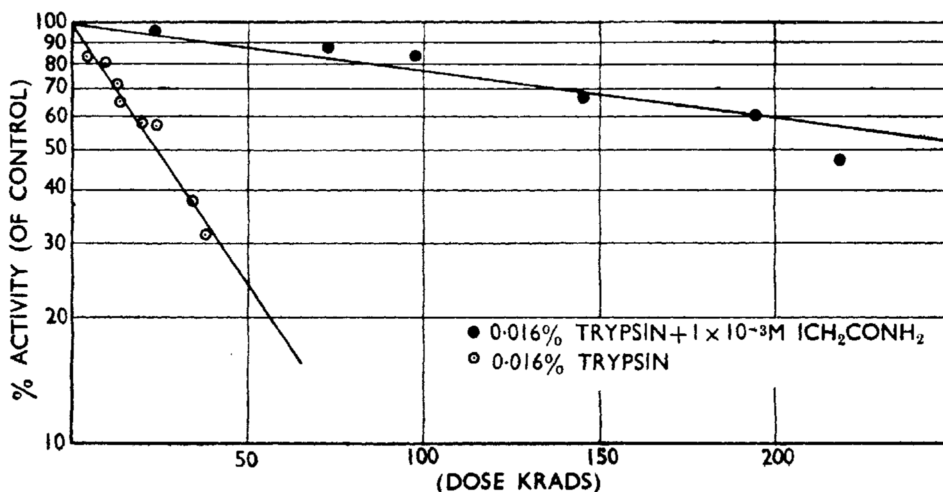


FIG. 4

better protector than any other additive. It is interesting to note that D_{37} values for the enzyme in the presence of KI and iodoacetamide are different, the latter offering a better protection due to reaction (5). However, it is worth while to point out that iodoacetamide also sensitizes bacterial systems and the same reactions are involved in this process (Mullenger *et al.* 1967).

Acknowledgement

The authors thank Prof. A. Charlesby and Dr. A. R. Gopal-Ayengar for encouragement and advice.

References

Anbar, M., and Levitzki, A. (1966). Copper-induced radiolytic deactivation of α-amylase and catalase. *Radiat. Res.*, **27**, 32–40.

Anbar, M., and Neta, P. (1965). Tables of bimolecular rate constants of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds. *Int. J. appl. Radiat. Isotopes,* **16**, 227–242.

Augenstein, L. G., and Grist, K. L. (1962). On the nature of enzyme inactivation by radiation. Biological effect of ionizing radiation at the molecular level. *I.A.E.A.*, Vienna, 263–273.

Braams, R. (1967). Rate constants of the hydrated electron reactions with peptides and proteins. *Radiat. Res.*, **31**, 8–26.

Brustad, T. (1966). The effects of radical scavengers on the radiosensitivity of lysozyme in dilute aqueous solutions of varying *p*H. *Radiat. Res.*, **27**, 456–473.

Gordon, S., Hart, E. J., Matheson, M. S., and Rabani, J. (1965). Reactions of hydrated electrons. *Disc of Faraday Soc.*, **36**, 193–205.

Hyder, M. L. (1965). The radiolysis of aqueous nitrate solutions. *J. Phys. Chem. Ithaca*, **69**, 1858–1865.

Mee, L. K., Navon, G., and Stein, G. (1964). Inactivation of chymotrypsin in aqueous solution by atomic hydrogen. *Nature, Lond.*, **204**, 1056-1057.

——— (1965). The action of atomic hydrogen on trypsin in aqueous solutions. *Biochem. biophys. Acta.*, **104**, 151–159.

Mullenger, L., Singh, B. B., Ormerod, M. G., and Dean, C. J. (1967). Chemical study of radiosensitization of *M. sodonensis* by iodine compounds. *Nature, Lond.*, **216**, 372–374.

Robins, A. B., and Butler, J. A. V. (1962). Effects of oxygen on the inactivation of enzymes by ionizing radiations. I. Dilute solutions of trypsin and deoxyribonuclease. *Radiat. Res.*, **16**, 7–17.

Sanner, T. (1965). Reduced pyridine coenzymes as hydrogen donors in repair of radiation damage. *Radiat. Res.*, **26**, 95–106.

Schwert, G. W., Neuraths, H., Kaufman, S., and Stoke, J. E. (1948). The specific esterase activity of trypsin. *J. Biol. Chem.*, **172**, 221–239.

Siddle, A. B. (1964). Relationship between chemical structure and the extent of protection against ionizing radiation given by alcohols to *Salmonella typhimurium LT2*. *Nature, Lond.*, **204**, 597-598.

Singh, B. B., Charlesby, A., Keene, J. P., and Swallow, A. J. (1966). Electron reaction with iodoacetamide. 3rd Int. Congr. of *Radiat. Res. Cortina D'Ampezzo.*