Russian Chemical Reviews **62** (5) 493 – 508 (1993)
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UDC 541.15

Radiation-induced inactivation of proteolytic enzymes

M A Orlova

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Abstract. Data on the mechanism of the inactivation of proteases under various conditions and the possible applications of these processes are surveyed. Serine, sulfhydryl, acid, and metal-containing proteases are considered. Attention is concentrated on the conformational changes in radiolytic processes: their dependence on the pH of the medium and the correlations with the change in the aminoacid composition of the enzymes. The bibliography includes 90 references.

I. Introduction

Ionising radiation occupies a special place in the series of influences on biological objects. This is associated, firstly, with the fact that there exists a natural radioactive background stimulating changes in the processes occurring on Earth. Secondly, the technological use of ionising radiation and nuclear processes has led to the possibility of the directed liberation of an enormous amount of energy and contamination of the ecosystem with the subsequent increase in the natural radioactive background and irradiation of the population with small doses; expressed quantitatively, this corresponds to an appreciable increase in the natural total annual irradiation dose received by the population, which up to 1986 was about 3×10^6 Gy person^{-1}.[1] It is evidently important to study such influences on the human organism as a whole and on its individual systems in order to discover the adaptive and protective mechanisms. The difference between ionising radiation and other physicochemical influences consists in the reduced wavelength (< 100 nm) and higher energy. The ionisation region begins with an energy in excess of 5 eV. The interaction of ionising radiation with matter, including biochemical objects, has been described in numerous monographs.[2-4] Without going into the details of the theoretical aspects of this process, we shall mention here only that the study of the indirect influence of ionising radiation through the radicals formed as a result of the radiolysis of the medium (in particular water) is more important and informative for biochemistry. The principal water radiolysis products are as follows:[5] H, OH, e_{aq}^-, O^{2-}, HO_2, H_2O_2. The radiation-chemical yield G (the amount of product formed per 100 eV of absorbed energy) depends on the presence of impurities (radical donors and acceptors), the pH of the solution, and other factors.[6]

The interaction of a biomolecule with these products leads to a change and degradation of structures, in particular those ensuring the functioning of the organism. The mechanisms of such processes have been described in another review for the simplest biomolecules (peptides, aminoacids, and proteins).[7]

The imbalance in the processes involving the activation and inactivation of enzyme systems, which in the case of low and chronic irradiation doses may not be manifested for a time but accelerates nevertheless the ageing of the systems of the organism, may be regarded as one of the ways in which the vital activity of an organism is disturbed. In view of this, there has been a growth of interest at the present time in the study of phenomena determining the relationship between the functioning of separate systems and combined influences, which presuppose the occurrence of synergism.[8] The necessity for such studies is associated with the sharp impairment of radioecology against the background of a disastrous ecological situation. The problem of discovering the consequences of the influence of small radiation doses as well as chemical reagents (radiomodifiers) on the results of radiolytic processes requires the creation of predictive models. Quite a large number have now been proposed — both purely biological and biochemical, including protease models.[9]

The development of radiotherapy is also closely related to problems of radiobiology and damage to individual portions of cells and microstructures of the organism.[10] This is partly a consequence of the use of new emitters (for example, neutron emitters[11,12] or emitters of beams of heavy ions[13,14]), which requires additional studies in the field of the relative biological effects of this influence on the principal components of the organism and different cells.

It has become evident in recent years that proteases play a decisive role in the processing of protein.[15] Many facts indicate that proteolytic enzymes perform the function of biological control in the organism, influencing objects important for life. A whole series of key metabolic enzymes are controlled directly by proteases. The mechanisms of the regulation of their activity can operate via the activation of zymogens, the inhibitor – protease balance, and the location of the protease with the substrate. It is therefore not surprising that, in the early stages of radiation and thermal lesions, changes were observed in proteolytic activity,[16] this finding being attributed to the pathology of the kallikrein – kinin system.

M A Orlova Department of Radiochemistry, Faculty of Chemistry, Lomonosov Moscow State University, Leninskie Gory, 119899 Moscow. Fax (7-095) 939 01 26; Tel. (7-095) 939 31 86

Received 4th February 1993
Uspekhi Khimii **62** (5) 529 – 544; translated by A K Grzybowski

The aim of the present review is a comprehensive examination of the mechanism of the radiolysis of proteases and the possible use of this process in medicine and bioengineering.

II. Irradiation of proteases in the solid and immobilised states

Irradiation of enzymes in the solid state constitutes the first stage in the study of the mechanism of their radiolysis, since it makes it possible to investigate the consequences of the direct action of radiation and not of the secondary products of the radiolysis of the medium. This also permits in many instances the determination of the relative molecular masses of both the individual enzymes and of complex biological formations. Studies on these lines, initiated in the 1950s,[17] have continued to this day as a result of a new assessment of the possibilities of the radiation method for the determination of relative molecular masses[18,19] and new computational methods.[20,21]

It is believed that, on irradiation of biochemical objects in the dry state, the site acted upon by the radiation is localised at the point of incidence, in contrast to solutions where the effective volume increases as a result of the diffusion of the water radiolysis products. Localisation is partly observed also on irradiation of frozen solutions,[22] although on subsequent heating to room temperature secondary reactions do occur. In all cases, the target theory is applicable[23] and the main quantitative characteristic of the process is the effect–dose relation, which is exponential for proteases. On the other hand, the deviations from the exponential function which are encountered permit inferences about the process mechanism, which may be complicated as a result of energy transfer in the biosystem.

On irradiation of an enzyme system, the energy transfer to the most radiosensitive component of the molecule can greatly increase the effective volume,[24] increasing it to the size of the entire biomolecule and this affects also the relative molecular masses determined. Since the inactivation of the system changes as a rule on raising the temperature, one should introduce a correction to the calculations of the molecular mass. This has been demonstrated.[25,26] The introduction of additives, i.e. irradiation of mixtures containing several components, also changes the degree of inactivation of the enzyme itself. For example, on irradiation of a mixture with glutathione[27] or another radioprotector, a decrease in the degree of inactivation of the enzyme is observed.

Theoretical problems concerning the determination of the sizes of complex equilibrium enzyme systems comprising several identical components[19] or identical functional units[20] have been examined.[19,20] In both cases, simple exponential effect–dose relations and more complex convex-concave curves may be observed, which depends on the oligomer–monomer equilibrium:

$$R + E \underset{}{\overset{K_d}{\rightleftharpoons}} RE ,$$

where R is the regulator unit, E the catalytic unit, and K_d the equilibrium dissociation constant.[9]

Depending on the size and concentrations of the components, the equilibrium constant, the relative activities of the free enzyme and the enzyme complex, and also the distribution of the components between the free and bound forms, the apparent size of the target and hence the relative molecular masses obtained also change during irradiation.

Analysis with the aid of the target theory likewise makes it possible to obtain an idea about the structural organisation of complex systems, which has been demonstrated in relation to a calmodulin-dependent phosphodiesterase[28] and the components of the adenylate cyclase system in erythrocytes.[29] The presence and the influence of the activator or inhibitor can also be determined from the form of the above relation.[20] In many instances it has been suggested that the role of the transfer of the energy of the oligomeric subunits be likewise estimated in this way when the dissociation of the oligomeric complex can be modelled.[20,28,30,31]

Examination of the form of the effect–dose relations and the application of the target theory on irradiation of a pure enzyme and enzyme complexes with different degrees of interaction of their components permit not only the development of a theoretical analysis but also the determination of the influence of the parameters on the cell populations[32] and also an understanding of the mechanism of the inactivation of a particular enzyme, which is itself a complex structural reacting unit.[33] The increase in the popularity of the method of radiation-induced inactivation for the determination of the dimensions of the systems is due to its simplicity, applicability to complex objects, and the possibility of using only partly purified preparations.

There are literature data on the inactivation of dry preparations of proteases: trypsin,[23,34–36] pepsin,[23] chymotrypsin, papain, subtilisin, and carboxypeptidase.[34,35] Two interrelated processes arise on irradiation: radical formation and inactivation. The radiation-chemical yield of radicals is $\sim 1-7$. γ-Irradiation yields information under these conditions about the distribution of carbon-based radicals (for example, in chymotrypsin at 195 K), preceding the secondary radicals observed at 295 K. The carbon-based radicals can be divided into two groups: those formed at a temperature below 195 K and independent of the aminoacid sequence and those formed above 195 K and depending on the conformation of a specific native enzyme.[37] The dose dependences are exponential and the inactivation reaction is first order for γ- and X-irradiation.

In order to investigate the damage caused in molecules by ionising radiation, it is convenient to employ subsequent tritiation, which has been used[38] to determine the changes which had occurred in α-chymotrypsin after γ-irradiation. On the basis of the aminoacid analysis of the tritiated specimens, the authors reached a conclusion about the conformational transformations, which entailed a change in the area of the accessible surface of the molecule and a greater importance of the hydrophobic residues.

In a number of studies, dry enzymes were irradiated in order to elucidate their radiation stabilities as such and to compare them with stabilities in relation to other methods of treatment. Figure 1 presents results characterising the loss of activity by certain dry proteases on irradiation.

It is noteworthy that the observed degree of inactivation of proteases may depend on the method of its determination, which hinders comparison of the results of different studies. In particular, this has been observed for chymotrypsin irradiated in a dry state.[35,36]

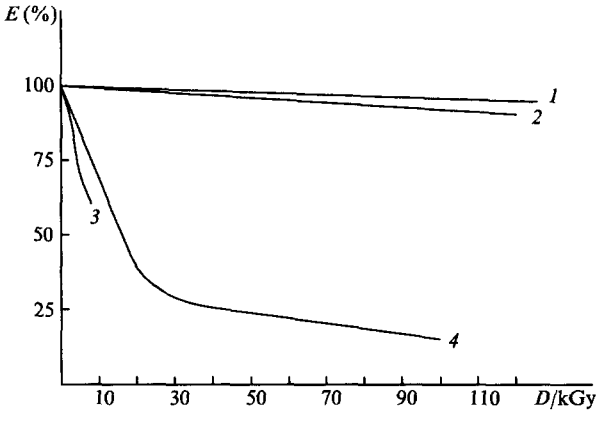


Figure 1. Radiation-induced inactivation of enzymes in the crystalline state: (*1*) α-chymotrypsin (X-rays);[39] (*2*) trypsin (γ-rays);[36] (*3*) glucosidase + catalase (X-rays);[39] (*4*) subtilisin-72 (X-rays).[39]

The stability of enzymes increases following their immobilisation and for this reason immobilised enzymes are actively employed in the national economy, especially in medicine. There exist three aspects of the study of the action of ionising radiation on immobilised enzymes. The first involves the use of ionising radiation for immobilisation itself. There exists an extensive literature on this question, which is not considered in the present review.

The second aspect involves the development of materials for the production of an image (for use in roentgenoscopy, autoradiography, and dosimetry). Thus α-chymotrypsin and subtilisin-72 have been bound[39] to various diazides and the vinylpyrrolidinone–acrolein copolymer on chromatographic paper in order to obtain a latent image and hence to detect X-radiation. The authors tested several versions of the method: solutions of the enzyme (2×10^{-3} M α-chymotrypsin, 1.1×10^{-3} M subtilisin) were deposited on a support and irradiated with X-rays at a dose rate of 200 rad s^{-1}; the enzyme and the diazide were deposited on a support and irradiated with X-rays transformed by a special screen into UV radiation at a wavelength of 365 nm; chromatographic paper was subjected to a preliminary treatment with *p*-azidobenzaldehyde diethyl acetal, the vinylpyrrolidinone–acrolein copolymer (VPA), or VPA modified by azide groups with subsequent exposure together with the enzyme. The subsequent development of the latent image by a suitable dye reveals a difference between the irradiated and nonirradiated sections. Exposure to X-rays of a carrier impregnated with an enzyme solution leads to the immobilisation of 0.05% of α-chymotrypsin and 0.01% of subtilisin.

The use of proteases for the preparation of dosimetric materials has been described.[40] Gelatin films containing small amounts of enzymes (chymotrypsin, papain, trypsin, and subtilisin) were exposed to the low-energy tritium β-radiation and development was carried out after irradiation in solutions of these enzymes. In both cases, irradiation hardly affected the activity of the enzyme but damaged the structure of gelatin, altering the degree of and conditions for its digestion by the enzyme. When the films are dyed, a half-tone image is formed and this method as well as the previous one can therefore be used to detect ionising radiation.

The third aspect of the studies involves an attempt to model the mechanism of the action of ionising radiation on immobilised systems via the investigation of the radicals generated. Such studies have been carried out for trypsin immobilised on dialdehyde-cellulose (DAC) by comparison with pure trypsin.[41,42] The range of γ-irradiation doses was 0.05–10 Mrad (0.5–100 kGy). Comparison of the EPR spectra of the immobilised and native trypsin as well as the support suggested that the increase in the stability of the immobilised trypsin is associated with the recombination of the trypsin radicals with the DAC radicals and with their migration to weakened bonds in the DAC. The activity of the immobilised trypsin does not then change, while native trypsin loses 25% of its activity for a dose of 10 Mrad (100 kGy).[42] Such increase in stability makes it possible to employ immobilised proteases in enzyme therapy using γ-sterilisation,[43] since the activity of γ-irradiated immobilised trypsin does not change during prolonged storage with a simultaneous decrease in the number of radicals. Trypsin immobilised on DAC can be stored under severe conditions for a long time. Comparison of the behaviour of the enzymes on γ-irradiation of their aqueous solutions and the enzymes immobilised on nylon fibres showed[44] that not all enzymes exhibit an appreciable increase in their resistance to radiation in the immobilised state; however, this does not apply to proteases.

III. The effect of ionising radiation on aqueous solutions of proteolytic enzymes

1. Serine proteases

The radiolysis of serine proteases in solution has been studied most fully. Questions associated with the mechanism of the radiation-induced damage to trypsin and chymotrypsin (the enzymes of the pancrease) and certain subtilisins (the enzymes from microorganisms produced by various strains of *B. subtilis*) have been examined in detail in the literature. The blood enzymes (thrombin and plasmin) have been much less investigated and as regards certain enzymes no information is encountered in the literature.

The main feature of the radiolytic damage to serine proteases is a fairly high stability of the active centres, containing a serine residue, in relation to the action of ionising radiation, whilst the conformational stability of the biomolecule is most vulnerable. It has been shown in a number of studies[45,46] that the main contribution to the inactivation of serine proteases comes from OH radicals obtained on radiolysis of water. This has been readily confirmed by irradiating chymotrypsin in N_2O, where the number of OH radicals increases as a result of the reaction

$$e_{aq}^- + N_2O + H_2O \rightarrow OH + OH^- + N_2 . \quad (1)$$

In this case, the corresponding increase in the radiation-chemical yield of the inactivation is observed.

The coefficients of susceptibility to damage (effectiveness of inactivation, i.e. the probability that the attack by a given active influence inactivates the enzyme) of chymotrypsin by various radicals, the number of which was regulated by controlling the irradiation conditions (Tables 1 and 2), have been published.[2,3] The rate constants for reactions with certain water radiolysis products are also known (Table 3). It has been shown that the changes induced by the OH and H radical species involve mainly tryptophan residues in chymotrypsin, while approximately 15%

Table 1. Radiation-chemical yields in radical formation (G_R) and inactivation (G_{in}) reactions in aqueous solutions of chymotrypsin and papain on γ-irradiation under different conditions.

Medium	Radicals	G_{in}	Effectiveness of inactivation, f	G_R	f/G_R
		Chymotrypsin[45]			
Air	OH	0.265	0.266	2.8	0.095
N_2O	2 OH, H	0.83	0.30	0.7	0.43
N_2O + *tert*-BuOH	H	0.091	0.091	0.7	0.13
Ar	OH, H	0.61	0.044	2.8 2.6	0.17
Ar + *tert*-BuOH	H	0.234	0.143	2.6	0.055
		Papain[47,48]			
N_2O	OH, H	–	–	5.9	0.43

Table 2. Effectiveness of the inactivation of enzymes by the water radiolysis products.

Reagent	Trypsin[75]	Chymotrypsin[45]
H	0.1	0.13
OH	0.05	0.137
e_{aq}^-	0.09	0.055
H_2O_2	0.04	–
HO_2	0.04	–
O_2	0.03	–

Table 3. The rate constants for the reactions of proteases with the water radiolysis products (γ-irradiation).

Substance	10^{-10} k/litre mol^{-1} s^{-1}			Ref.
	e^-_{aq}	OH	O_2^-	
Proteins	5	1–	$6<10^{-7}$	23
Chymotrypsin	–	3.7±0.4	–	49
Papain	4.1	4.7	–	48

of e^-_{aq} react at the S–S bonds. The next stage of the study was therefore the determination of the relationship between the damage to the tryptophan residues and the conformational stability.

A detailed analysis has been carried out of the mechanism of the inactivation of chymotrypsin.[49-51] A change in the accessible surface of the globule was detected by the $^1H-^3H$ exchange method.[46,50] The rate of exchange increased by 16% compared with the nonirradiated enzyme with increase in the irradiation dose from 30 to 50 Gy. The use of fluorescence, viscometric, and microcalorimetric methods made it possible to determine the radiation-chemical yield of the uncoiled molecules (G_{unc}) and to find the correlation between G_{unc} and the radiation-chemical yield of the damaged tryptophan residues $-G(-Trp)$[49] (Table 4). Data on the binding of proflavin[53] demonstrated the presence of local steric changes around the binding centres. Conformational changes were initially detected only after an irradiation dose of about 2 kGy,[46,49] but later they were discovered also at lower doses (< 1 kGy).[50,51] The change in the fluorescence quantum yield in irradiated chymotrypsin solutions, characterising the number of tryptophan residues, showed[52] that at pH 3 their decrease is correlated with the decrease in catalytic activity. At pH 7.8 (the pH optimum of the esterase activity), an increase in the number of accessible tryptophan residues was observed initially, decreasing following an increase in their dose. These data make it possible to infer the changes in the degree of uncoiling of the chymotrypsin globule as a function of pH. In particular, it appears that at low pH the area of the accessible surface of the globule is greater. The maximum change in this quantity was observed at pH 2.0, whereupon the radiation-chemical yield of the inactivation reaction increases.[53] For doses in excess of 0.6 kGy, the conformational changes became insignificant but the aminoacid composition of the enzyme changed sharply.[51]

Damage to the disulfide bridges occurred at higher irradiation doses and no longer played a significant role in the breakdown of catalytic activity. Thus, for a dose of 0.5 kGy, 5.5 S–S bonds were detected in chymotrypsin,[50] while for a dose of 0.8 kGy the number of such bonds was 4. It was found that the presence of S–S bonds is important for reducing the conformational changes on irradiation. This was demonstrated by comparing the γ-inactivation of chymotrypsin and subtilisin,[52] where the same degree of inactivation in subtilisin entails the decomposition of a smaller number of important aminoacid residues. This conclusion follows from the comparison of the ratios $G(-Trp)/G_{in}$. This quantity is 4.3 times smaller for subtilisin than for chymotrypsin, while G_{in} (subtilisin) is smaller than G_{in} (chymotrypsin) by a factor of 1.4 (Table 4). A similar difference has been observed also between the overall quantities $G(Tyr+Trp)/G_{in}$. The authors see an explanation of this effect in the presence of five S–S bonds in chymotrypsin (subtilisin is a single-stranded enzyme and has no disulfide bridges), which, as it were, 'maintain' the structure of the enzyme under the conditions where the tryptophan residues are damaged.

The change in the aminoacid composition of chymotrypsin for different irradiation doses has been investigated in a number of studies.[50-52] $5\times10^{-6}-10^{-5}$ M solutions at pH 3 (HCl)[50] and 7.8 (tris-HCl buffer)[52] were used. After irradiation with a dose of 0.5 kGy, a decrease in the number of tyrosine residues and, after a dose of 1.2 kGy also, in phenylalanine residues was observed together with the damage to the tryptophan residues.[50] For a dose of about 1.6 kGy, almost all the aminoacid residues are involved. The aminoacid compositions[52] of the irradiated solutions at pH 3 and 7.8 were compared. A common feature of these compositions is a decrease in the number of tyrosine, cysteine, phenylalanine, and lysine residues, but the doses required for this are smaller at pH 3. Thus the decomposition of one tyrosine residue requires about 0.025 kGy at pH 3 and approximately 0.6 kGy at pH 7.8. A greater radiation yield G_{decomp} for chymotrypsin at pH 3 compared with pH 6.9 has been observed.[49] Comparison of data from different studies shows that, by virtue of their more uncoiled conformation, the damage to the aminoacid residues on irradiation with high doses at pH 3 is stochastic and evidently the change in conformation is more important for catalytic activity than the change in the aminoacid composition (except for the aromatic aminoacid residues).

A correlation between the decrease in catalytic activity and the decomposition of tyrosine residues has been observed for irradiated subtilisin-BPN′ and subtilisin-72,[52,54] especially at pH 8.5 (the pH optimum of the activity of the enzyme).[52] At lower pH (for example, 5.5), this correlation is less marked, since there is a dependence on the decomposition of tryptophan residues. The degradation of the histidine residues forming part of the active centre of subtilisin-72 is observed at doses in excess of 5 kGy, whereupon the catalytic activity is almost completely lost. Histidine is more important for the radiation stability of subtilisin-BPN′. After γ-irradiation with a dose of 1.1 kGy, subtilisin-BPN′ loses two tyrosine residues and one tryptophan, one methionine, and one histidine residues[53,54] (Figure 2). The reaction with diisopropyl phosphorofluoridate shows that under these conditions 85% of the active centres are retained. For an irradiation dose of 2.4 kGy, changes appear in the leucine, proline, phenylalanine, serine, and alanine residues. The

Table 4. Comparison of the radiation-chemical yields in the uncoiling of enzyme molecules (G_{unc}) and inactivation (G_{in}) with the radiation-chemical yield in the decomposition of aminoacid residues.

Enzyme	Irradiation conditions	G_{unc}	G_{in}	$G(-Trp)$	$G(-Tyr)$	$G(-Trp)/G_{unc}$	$G(-Tyr)/G_{unc}$	Ref.
Trypsin	HCl, pH 3.0	0.37	–	0.9	–	2.4	–	49
Chymotrypsin	HCl, pH 3.0	0.43	–	1.2	–	2.8	–	49
Trypsin	H_2O, pH 6.6	0.32	–	0.7	–	2.2	–	49
Chymotrypsin	H_2O, pH 6.6	0.31	–	1.1	–	3.5	–	49
Trypsin	Glycine buffer, pH 6.9	0.26	–	0.6	–	2.3	–	49
Chymotrypsin	Glycine buffer, pH 6.9	0.18	–	0.6	–	2.5	–	49
Trypsin	Phosphate buffer	0.36	–	0.9	–	2.5	–	49
Chymotrypsin	Phosphate buffer	0.30	–	0.9	–	3.0	–	49
Subtilisin-72	0.1 M NaCl, pH 5.6	–	0.23	0.19	0.35	0.82	1.5	52
	0.1 M NaCl + NaOH, pH 8.5	–	0.29	0.20	0.55	0.69	1.9	52
	Acetate buffer, pH 5.0	–	0.19	0.22	0.73	1.2	3.9	52

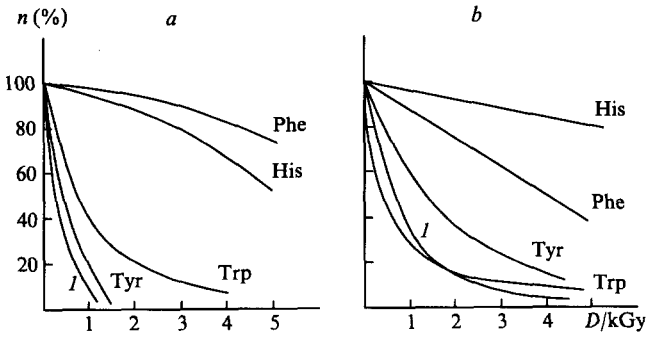


Figure 2. Variation of the number of aminoacid residues (n) in subtilisin-72 with the irradiation dose: (*a*) pH 8.5, 0.1 M NaCl + NaOH; (*b*) pH 5.0, 5×10^{-3} M acetate buffer; (*1*) variation of catalytic activity.

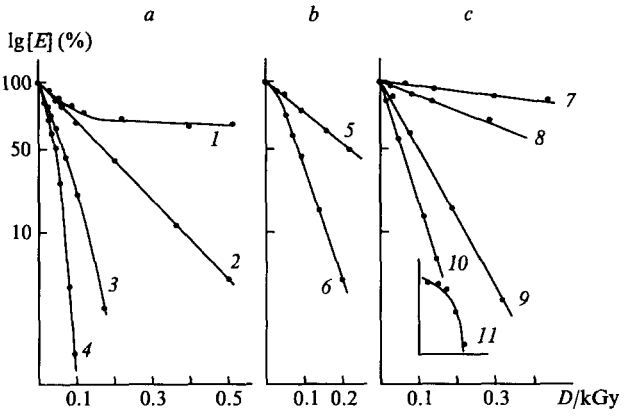


Figure 3. Dependence of the change in the catalytic activity of enzymes on the absorbed γ-radiation dose:[33] (*a*) α-chymotrypsin; (*b*) trypsin; (*c*) subtilisin-72; concentration (M): (*1*) 10^{-4}; (*2*) 7.5×10^{-6}; (*3*) 10^{-6}; (*4*) 10^{-7}; (*5*) 10^{-5}; (*6*) 10^{-6}; (*7*) 5×10^{-5}; (*8*) 5×10^{-6}; (*9*) 9×10^{-7}; (*10*) 3×10^{-7}; (*11*) 8×10^{-8}.

oxidation of methionine does not then lead to the inactivation of subtilisin-BPN',[54] but is significant for subtilisin-Carlsberg,[55] which differs, according to the data of Alden et al.,[56] from subtilisin-BPN' by the presence of a series of repeating segments in the polypeptide chain. For a dose of 0.55 kGy, the number of degraded tyrosine residues increases in subtilisin-72 compared with subtilisin-BPN', but the histidine residue is not degraded.[52] For a dose of 1.1 kGy, the difference between the numbers of degraded tyrosine residues increases still further. These data led to the conclusion that the tyrosine residues are more accessible in subtilisin-72.

Application of the electrophoretic method to irradiated subtilisin-BPN' demonstrated[53] the presence of a larger electric charge compared with the native enzyme, accompanied by the absence of changes in catalytic activity. This agrees with the earlier hypothesis that small irradiation doses lead to the formation of noninactivated molecules, which, however, differ conformationally from the native ones.

The appearance of active but partly damaged molecules has been confirmed by a change, even for low irradiation doses, in the kinetic parameters — the Michaelis constant (K_M) and the maximum rate of the catalytic reaction (V_{max}). Irradiation of dilute solutions increases K_M and diminishes V_{max} both in the case of chymotrypsin[57,58] and subtilisin-BPN' (Table 5).[54] Since the main damage to serine proteases caused by low doses is conformational damage associated with the disappearance of tryptophan residues (at least in chymotrypsin) and since in ribonuclease, which contains no tryptophan residues, there are no changes in K_M and V_{max},[59] the modification of the molecules is attributed to changes in the tryptophan residues. Other workers arrived at similar conclusions on the basis of the results of the irradiation of chymotrypsin and chymotrypsinogen.[60] The presence of modified molecules is reflected in the nature of the effect–dose relation, in particular deviations of the exponential function appear and the form of the curve depends on the initial enzyme concentration (Figure 3). A change in the radiation-chemical yield of the inactivation as a function of the initial enzyme concentration [E_0] has been observed[61–64] for trypsin and chymotrypsin (Table 6). This relation was described by the equation $G_{in} \simeq [E]^N$, where N is an integer. However, there is no concentration dependence if the active radicals (in this case OH radicals) react with the dissolved enzyme molecules faster than with one another, so that the decisive factor is the ratio of the number of the dissolved molecules to the number of active radicals. For trypsin, chymotrypsin, and subtilisin-72, the concentration ranges in which the dose dependences follow the exponential law have been determined:[33] $3 \times 10^{-6} - 5 \times 10^{-5}$ M for chymotrypsin, $2 \times 10^{-6} - 10^{-4}$ M for subtilisin-72, and above 10^{-6} M for trypsin. At lower concentrations, a deviation towards increasing inactivation is observed, whilst at higher concentrations ($> 10^{-4}$ M for chymotrypsin) an increase in dose

Table 5. The change in the kinetic parameters of irradiated proteolytic enzymes.

Enzyme	Irradiation conditions	Radiation	Dose/kGy	Substrate	Number of active centres	K_M/M	V_{max}/mol min^{-1} mg^{-1} [c]	Ref.
Subtilisin-72	0.1 M KCl, 30 °C, pH 7.5	γ	–	ATEE [a]	1.00	4.9×10^{-2}	7.6×10^2	54
	0.1 M KCl, 30 °C, pH 7.5	γ	0.95	ATEE	0.86	5.6×10^{-2}	7.6×10^2	54
	0.1 M KCl, 30 °C, pH 7.5	γ	1.66	ATEE	0.77	6.1×10^{-2}	6.6×10^2	54
Subtilisin-BPN'	0.1 M KCl, 30 °C, pH 7.5	γ	–	BTEE [b]	1.00	1.03×10^{-2}	3.46×10^2	54
	0.1 M KCl, 30 °C, pH 7.5	γ	1.2	BTEE	0.83	1.01×10^{-2}	2.66×10^2	54
	0.1 M KCl, 30 °C, pH 7.5	γ	1.66	BTEE	0.77	1.01×10^{-2}	2.08×10^2	54
α-Chymotrypsin	H_2O, 0 °C, pH 5.0, 60 mg ml^{-1}	X	–	ATEE	–	3.5×10^{-3}	100%	57
	H_2O, 0 °C, pH 5.0, 60 mg ml^{-1}	X	0.05	ATEE	–	5.2×10^{-3}	83%	57
	H_2O, 0 °C, pH 5.0, 60 mg ml^{-1}	X	–	ATEE	–	2.8×10^{-3}	100%	57
	H_2O, 0 °C, pH 5.0, 60 mg ml^{-1}	X	0.5	ATEE	–	5.4×10^{-3}	77%	57
	H_2O, 0 °C, pH 5.0, 60 mg ml^{-1}	X	0.75	ATEE	–	6.9×10^{-3}	66%	57

[a] ATEE = ethyl ester of *N*-acetyl-L-tryosine.
[b] BTEE = ethyl ester of *N*-benzoyl-L-tyrosine.
[c] V_{max} for α-chymotrypsin is expressed as a percentage of V_{max} for the nonirradiated enzyme.

Table 6. Dependence of the catalytic activity of serine proteases in aqueous solutions on the irradiation conditions.

Enzyme	Irradiation conditions	t/°C	Radiation	Dose rate/ Gy s^{-1}	[E]/M	Substrate	Dose/ Gy	D_{37}	G_{in}	G_{conf}	Residual enzyme activity (%)	Ref.
Trypsin	3×10^{-3} M H_2SO_4	2	X	0.075	4×10^{-3}	Haemoglobin	25.2	–	–	–	0	64
	3×10^{-3} M H_2SO_4	25	X	0.075	4×10^{-3}	Haemoglobin	25.2	–	–	–	49	64
	3×10^{-3} M H_2SO_4	37	X	0.075	4×10^{-3}	Haemoglobin	25.2	–	–	–	29	64
	5×10^{-3} M HCl, pH 3.0, air	20	X	0.075	2×10^{-4}	Haemoglobin	–	–	1.22 M/1000 r	–	–	63
	5×10^{-3} M HCl, pH 3.0, air	20	X	0.075	10^{-7}	Haemoglobin	–	–	0.057 M/1000 r	–	–	63
	H_2O air	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	86	62
	10^{-3} M phosphate buffer, pH 6.1	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	83	62
	10^{-2} M phosphate buffer, pH 6.1	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	80	62
	10^{-1} M phosphate buffer, pH 6.1	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	69	62
	2.5×10^{-3} M acetate buffer, pH 4.1	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	50	62
	5×10^{-2} M acetate buffer, pH 4.1	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	15	62
	10^{-3} M universal buffer, pH 5.3	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	5	62
	5×10^{-3} M universal buffer, pH 5.3	20	X	0.075	10^{-3}	Haemoglobin	100	–	–	–	4	62
	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-tyrosine	–	17.5	–	–	–	65
	HCl, pH 3.0, N_2	20	X	0.095	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-tyrosine	–	8.8	–	–	–	65
	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-leucine	–	8.3	–	–	–	65
	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	BTEE	–	21.8	–	–	–	65
	HCl, pH 3.0, N_2	20	X	0.095	5×10^{-6}	BTEE	–	8.2	–	–	–	65
	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	Methyl ester of *p*-toluene-sulfonyl-L-arginine	–	22.2	–	–	–	65
	HCl, pH 3.0, N_2	20	X	0.095	5×10^{-6}	Methyl ester of *p*-toluene-sulfonyl-L-arginine	–	8.3	–	–	–	65
	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	*p*-Nitroanilide of benzoyl-DL-arginine hydrochloride	–	18.2	–	–	–	65

Trypsin	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	*p*-Nitroanilide of benzoyl-DL-arginine hydrochloride	–	8.0	–	–	–	65
	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	β-Naphthylamide of benzoyl-DL-arginine hydrochloride	–	10.3	–	–	–	65
	HCl, pH 3.0, N_2	20	X	0.095	5×10^{-6}	β-Naphthylamide of benzoyl-DL-arginine hydrochloride	–	5.8	–	–	–	65
	HCl, pH 3.0, air	20	X	0.095	5×10^{-6}	Bovine serum albumin	–	13.1	–	–	–	65
	HCl, pH 3.0, N_2	20	γ	0.095	5×10^{-6}	Bovine serum albumin	–	6.0	–	–	–	65
	HCl, pH 3.0, air	20	γ	0.43	2×10^{-5} – $- 4 \times 10^{-4}$	Fluorescence of tryptophan residues	–	–	–	0.37	–	49
	H_2O, pH 6.6, air	20	γ	0.43	2×10^{-5} – $- 4 \times 10^{-4}$	Fluorescence of tryptophan residues	–	–	–	0.32	–	49
	Glycine buffer, pH 6.9, air	20	γ	0.43	2×10^{-5} – $- 4 \times 10^{-4}$	Fluorescence of tryptophan residues	–	–	–	0.26	–	49
	Phosphate buffer, pH 6.9	20	γ	0.43	2×10^{-5} – $- 4 \times 10^{-4}$	Fluorescence of tryptophan residues	–	–	–	0.36	–	49
Chymotrypsin	3×10^{-3} M H_2SO_4, air	20	X	0.075	2.8×10^{-4}	Haemoglobin	25.2	–	0.87 M/1000 r	–	–	61
	3×10^{-3} M H_2SO_4, air	20	X	0.075	6.4×10^{-6}	Haemoglobin	25.2	–	0.5 M/1000 r	–	–	61
	HCl, pH 3.0, air	37	X	17	2×10^{-4}	Casein	–	1.8×10^{4}	–	–	–	60
	HCl, pH 3.0, N_2	37	X	17	2×10^{-4}	Casein	–	9.5×10^{3}	–	–	–	60
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	ATEE	–	150	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	BTEE	–	170	–	–	–	50
	HCl, pH 3.0, N_2	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	BTEE	–	117	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	Methyl ester of *N*-acetyl-L-tyrosine	–	155	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	Ethyl ester of L-tyrosine	–	136	–	–	–	50

Table 6 (continued)

Enzyme	Irradiation conditions	t/°C	Radiation	Dose rate/ Gy s^{-1}	[E]/M	Substrate	Dose/ Gy	D_{37}	G_{in}	G_{conf}	Residual enzyme activity (%)	Ref.
Chymotrypsin	HCl, pH 3.0, N_2	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	Ethyl ester of L-tyrosine	–	99	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-tyrosine	–	140	–	–	–	50
	HCl, pH 3.0, N_2	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-tyrosine	–	68	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-leucine	–	171	–	–	–	50
	HCl, pH 3.0, N_2	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-leucine	–	84	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-tryptophan	–	140	–	–	–	50
	HCl, pH 3.0, N_2	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	*p*-Nitrophenyl ester of *N*-benzyloxy-carbonyl-L-tryptophan	–	76	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	Glutaryl-L-phenylalanine β-naphthylamide	–	105	–	–	–	50
	HCl, pH 3.0, N_2	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	Glutaryl-L-phenylalanine β-naphthylamide	–	54	–	–	–	50
	HCl, pH 3.0, air	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	Glutaryl-L-phenylalanine *p*-nitroanilide	–	147	–	–	–	50
	HCl, pH 3.0, N_2	20	X, γ	0.1 (P_X) 0.095 (P_γ)	5×10^{-6}	Glutaryl-L-phenylalanine *p*-nitroanilide	–	76	–	–	–	50
	10^{-3} M phosphate buffer, pH 7, air	20	γ	0.12	1.6×10^{-6}	ATEE	–	45.9	0.265	–	–	45
	10^{-3} M phosphate buffer, pH 7, argon	20	γ	0.12	1.6×10^{-6}	ATEE	–	20	0.61	–	–	45
	10^{-3} M phosphate buffer, pH 7, N_2O	20	γ	0.12	1.6×10^{-6}	ATEE	–	14.5	0.83	–	–	45
	HCl, pH 3.0, air	20	γ	0.43	2×10^{-5} – 4×10^{-4}	Fluorescence of tryptophan residues	–	–	–	0.43	–	66
	H_2O, pH 6.2, air	20	γ	0.43	2×10^{-5} – 4×10^{-4}	Fluorescence of tryptophan residues	–	–	–	0.31	–	66

Chymotrypsin	Glycine buffer, pH 6.9	20	γ	0.43	2×10^{-5} – -4×10^{-4}	Fluorescence of tryptophan residues	–	–	–	0.18	–	66
	Phosphate buffer, pH 6.9	20	γ	0.43	2×10^{-5} – -4×10^{-4}	Fluorescence of tryptophan residues	–	–	–	0.30	–	66
	Phosphate buffer, pH 6.9	20	X	–	1.6×10^{-5}	Casein	–	40	–	–	–	65
	Phosphate buffer, pH 6.9	20	X	–	8×10^{-5}	Casein	–	50	–	–	–	65
	Phosphate buffer, pH 6.9	20	X	–	1.6×10^{-5}	ATEE	–	20	–	–	–	65
	Phosphate buffer, pH 6.9	20	X	–	8×10^{-5}	ATEE	–	40	–	–	–	65
	tris-HCl buffer, pH 7.8, air	20	γ	0.058	10^{-5}	ATEE	–	–	0.44	–	–	67
	tris-HCl buffer, pH 7.8, air	20	γ, *n*	0.55×10^{-4}	10^{-5}	ATEE	–	–	0.40	–	–	67
Subtilisin-BPN′	H_2O, pH 6, air	2	γ	0.125	0.42×10^{-4}	ATEE	550	–	–	–	76	53
	H_2O, pH 6, air	2	γ	0.125	0.42×10^{-4}	ATEE	550	–	–	–	84	53
	H_2O, pH 6, air	2	γ	0.125	0.42×10^{-4}	ATEE	550	–	–	–	98	53
Subtilisin-72	0.1 M NaCl, pH 5.5, air	20	γ	0.083	10^{-4}	ATEE	–	–	0.17	–	–	68
	5×10^{-3} M acetate buffer, pH 5, air	20	γ	0.083	10^{-4}	ATEE	–	–	0.15	–	–	68
	5×10^{-3} M acetate buffer, pH 5, air	20	γ	0.083	10^{-5}	ATEE	–	–	0.06	–	–	68
	5×10^{-3} M acetate buffer, pH 5, air	20	γ	0.083	5×10^{-6}	ATEE	–	–	0.05	–	–	68
	5×10^{-3} M acetate buffer, pH 5, air	20	γ	0.083	1×10^{-6}	ATEE	–	–	0.01	–	–	68
	0.1 M NaCl, pH 5.5, air	20	γ	0.083	1×10^{-5}	ATEE	–	–	0.12	–	–	68
	0.1 M NaCl, pH 5.5, air	20	γ	0.083	5×10^{-6}	ATEE	–	–	0.10	–	–	68
	0.1 M NaCl, pH 5.5, air	20	γ	0.083	1×10^{-6}	ATEE	–	–	0.09	–	–	68
Carboxy-peptidase A	N_2	20	γ	–	–	Haemoglobin	–	–	0.27	–	–	69
	N_2O	20	γ	–	–	Haemoglobin	–	–	0.44	–	–	69
	O_2	20	γ	–	–	Haemoglobin	–	–	0.26	–	–	69
	N_2O + *tert*-BuOH	20	γ	–	–	Haemoglobin	–	–	0.11	–	–	69

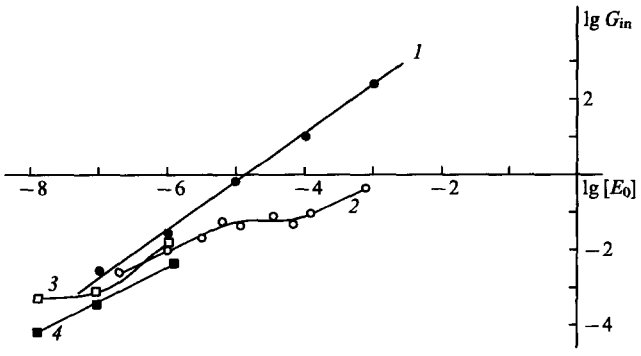


Figure 4. Dependence of the radiation-chemical inactivation yield (G_{in}) on the initial concentrations of chymotrypsin (curves *1* and *2*) and the angiotensin converting enzyme (curves *3* and *4*). For curve *1*, G_{in} was calculated along the initial section of the dose dependence plot and for curve *2* at a dose of 200 Gy; pH: (*1*) and (*2*) 7.8; (*3*) 7.5; (*4*) 9.0.

actually entails a decrease in inactivation compared with the expected value. The simplest calculation of the ratio of the active radicals derived from water and the enzyme molecules for a dose of 20 Gy demonstrated a relation between the observed deviations and the excess of either OH radicals ($< 10^{-6}$ M) or enzyme molecules ($> 10^{-5}$ M). Although the purity of the enzyme and also its possible immobilisation on glass can in fact alter the absolute value of G_{in} under these conditions, they do not affect the nature of the dose dependence. The curve relating G_{in} to the initial enzyme concentration is presented in Figure 4 in terms of logarithmic coordinates. For low doses, it is linear but for a dose of 0.2 kGy there is already a plateau in the chymotrypsin concentration range $5 \times 10^{-6} - 6 \times 10^{-4}$ M. The authors therefore suggest that two inactivation regions, linked to the relation examined above, be distinguished: (1) the number of inactivated molecules is proportional to the initial enzyme concentration (a dose < 20 kGy in the case of chymotrypsin), i.e. a definite fraction of the molecules are inactivated; (2) the number of inactivated molecules ceases to depend on the initial concentration. Very low enzyme concentrations ($< 10^{-7}$ M), at which the excess of radicals is too high, and concentrations above 10^{-3} M, where the direct action of the radiation on the energy transfer process may become important as a result of aggregation, are possible exceptions.

The method used to determine the enzyme activity has a major influence on the value of G_{in} and the form of the effect–dose relation. Lynn[65] pointed out the absence of an exponential dose dependence in the case of trypsin when the catalytic activity was determined with the aid of casein and put forward an explanation based on the methodological characteristics of the determination, namely on the differences between the rates of the tryptic and chymotryptic digestion of the peptides formed.[71] The mixture of peptides varied depending on the degree of irradiation, and its solubility in trichloroacetic acid, used in this method, also varied. As already pointed out above, there are deviations from the exponential relation also when ester substrates are used, in particular, the ethyl ester of *N*-acetyl-L-tyrosine (ATEE).[33,65,71] The reason for and the extent of these differences have been investigated in numerous studies devoted to chymotrypsin,[33,60,65,71,72] trypsin,[65] and subtilisin-BPN′,[53] mainly in relation to casein and ATEE (Table 6). Substrates can be arranged in the following sequence in terms of increasing sensitivity: casein ≪ BTEE < ATEE. This fact confirms the conformational changes on irradiation. By virtue of the difference in the size of the biomolecules, the smallest conformational change affects the ability of the enzyme molecules to bind different substrates. Furthermore, casein apparently has several centres for binding to the enzyme by virtue of its structure, whereas in esters there is only one such centre. The difference between the specificities of ester substrates can be explained by different binding mechanisms.[53] Thus nine ester substrates have been used[72] and it has been shown that for some of them there is a relation between the loss of catalytic activity and the decomposition of the tryptophan derivatives, but this is not true of all substrates. Under these conditions, both catalytic stages — acylation of the active centre as well as deacylation — are acted upon by ionising radiation.

It is noteworthy that in all cases the purities of the enzyme and the substrate are very important for the sensitivity to radiation; this has been demonstrated specially in relation to casein.[65]

The reaction conditions, i.e. the composition of the medium, alter the radiation-chemical yield of active radicals and influence greatly G_{in}. The influence of different buffers on the latter is indicated in Table 6 and for subtilisin-72 has been described in a study[68] where the dependences of G_{in} on the initial enzyme concentration in the acetate buffer and in 0.1 M NaCl solution were compared. At a high enzyme concentration (10^{-4} M), the difference is insignificant, but in 10^{-6} M solution it already increases almost by an order of magnitude. The greatest effect of the acetate buffer on trypsin has been observed by McDonald.[62] On the other hand, the tris-HCl buffer hardly affected G_{in} for chymotrypsin.[51]

The presence of Ca^{2+} ions is important under irradiation. There are data indicating their binding to enzyme molecules (particularly those of the subtilisins), which leads to the observation of a protective effect, G_{in} diminishing for serine proteases as a result of the decrease in G_{unc}. It has been shown[51] that the presence of Ca^{2+} retards the decomposition of the tryptophan residues. The stabilising effect of Ca^{2+} is known also in thermal inactivation.[73] According to Nikol'skaya and coworkers,[51,68] the stabilising effect increases with increase in enzyme concentration; D_{50} (the dose at which 50% of the active enzyme remains) increases by a factor of 2. The stability increases also with increase in pH to 7.8 (D_{50} increases by a factor of 1.5 for a concentration of 10^{-6} M and by a factor of 3 for 10^{-4} M). The joint influence of calcium ions and the acetate buffer has been investigated for subtilisin-72.[68] In this case, competing processes are observed and the protective effect of Ca^{2+} ions is manifested when the effect due to the binding of Ca^{2+} to subtilisin molecules begins to exceed the destabilising effect of the increase in the ionic strength of the solution. There is a region in which the effects cancel out (in the given instance at an enzyme concentration of about 3×10^{-6} M).

The change in the inactivation of chymotrypsin and subtilisin as a function of the type of irradiation has been described[59,67] and is illustrated in Table 7. However, one should bear in mind that such variation depends as a rule on the dose rate of the source, which also influences the radiation stability of enzymes.

Table 7. Dependence of the inactivation constant ($k_{in,eff}$) on the type of radiation.[67]

Enzyme	Medium	pH	Radiation	$10^4\ k_{in,eff}$/min^{-1}
Chymotrypsin, 10^{-5} M	Tris-HCl buffer	7.8	γ	1.0
	Tris-HCl buffer	7.8	γ-n[a]	0.5
Subtilisin-72, 10^{-6} M	0.1 M NaCl	5.5	γ	0.9
	0.1 M NaCl	5.5	γ-n[a]	0.5

[a] Gamma-neutron field.

Many questions are associated with the influence of oxygen on the radiation-induced inactivation process. The results of the irradiation of serine proteases in air and in an inert gas atmosphere have been compared,[50,60] but the conclusions are not unambiguous. It is assumed[65] that, on irradiation in air, the principal role is assumed by OH radicals, whilst under anaerobic conditions it is assumed by both OH and H. According to the

results of Masuda et al.,[74] the yields $G(-SH)$ and $G(-Trp)$ are smaller under anaerobic conditions, whilst in the case of chymotrypsin a greater sensitivity was observed in the absence of oxygen.[57] One of the explanations of differences of this kind may be provided by the dependence on the medium in which the irradiation is carried out (the same competing processes involving the interaction of the enzyme with the active radicals and of the radicals with one another). Thus O_2^- ions are formed faster at pH 5.0 than H atoms and the effectiveness of the inactivation by them is much smaller.[57] The protective role of oxygen on irradiation in air has been observed.[72] In many instances the formation of H_2O_2 is decisive. This has been demonstrated in relation to subtilisin-Carlsberg.[55]

All the data considered above refer to the study of the enzymes *in vitro*. Another version of the study of the changes in the function of the enzyme under influence of ionising radiation constitutes studies *in vivo*. Investigations of this kind[75,76] have been performed to determine the changes in the fibrinolytic metabolism associated with the activity of plasmin. It has been shown that the fibrinolytic activity diminishes with increase in the γ-irradiation dose *in vivo* and this is associated in its turn with the decrease in the reactivity of the plasminogen activator. Unfortunately it was not possible to find any literature data on the mechanism of the radiolysis of the enzymes thrombin and plasmin themselves, but one can postulate that it is common to the serine proteases and involves a change in the conformation of the active centre.

2. Sulfhydryl proteases

Proteases containing cysteine in the active centres are of special interest in the study of their radiosensitivity in connection with the hypothesis of a unique mechanism of radiation sickness associated with the number of free sulfhydryl groups in the organism.[77] The radiolysis of one of the most thoroughly investigated sulfhydryl proteases (papain) has been examined in detail.[78,79] As already pointed out above, the majority of studies on the radiolysis of enzymes have been carried out in the region where the exponential effect–dose relation holds, the latter being as a rule a result of the combination of several nonexponential processes. The kinetics of the inactivation of papain have been described[78] and the following relation between the dose and the degree of inactivation has been obtained:

$$D\alpha = \frac{k' - k_1}{k_0} y + \frac{k_1 E_0 + k'_1 F + k_2 P}{k_0} \ln \frac{E_0}{E_0 - y}, \qquad (2)$$

where D is the dose in Roentgens, α the proportionality coefficient which converts the dose into the number of radicals ($It = \alpha D$), I the number of radicals (mol litre^{-1}) formed per unit time, k' the rate constant for the inactivation of the radicals by the active enzyme, k_1 the rate constant for the inactivation of the radicals by the inactivated enzyme, k_0 the rate constant for the inactivation of the enzyme by the radicals, E_0 the initial enzyme concentration, k'_1 the rate constant for the inactivation of the radicals by contaminants, F the concentration of contaminants in the enzyme, k_2 the rate constant for the inactivation of the radicals by radioprotectors, y the concentration of the inactivated enzyme, and P the concentration of the radioprotectors.

Eqn (2) is in principle applicable to any enzyme. It has linear and logarithmic components. Only when $k' = k_1$, i.e. if the active and inactivated enzymes react with the radicals in the same way, does the exponential relation hold for the dose–effect curves, since we then have

$$E = E_0 \exp(-k_0 D\alpha / [k_1 E_0 + k'_1 F + k_2 P]) = \\ = E_0 \exp(-KD). \qquad (3)$$

However, the authors observed that, for an exponential dose dependence of the X-inactivation of papain, the active enzyme molecules react with the water radiolysis products faster than the inactivated molecules. This made it necessary to examine in greater detail the inactivation kinetics. A family of curves was obtained with an increase in the inactivation yield as a function of the dose, which is associated with the decrease in the importance of the logarithmic part of Eqn (2) as k_0 increases. The theoretical ideas agreed well with the experimental data for the X-inactivation of 1.2×10^{-5} M solutions of papain in phosphate buffer at pH 7.4 and 0 °C. When $k' \neq k_1$, the radiosensitivity parameters change, which usually diminishes the degree of inactivation. Gaucher et al.[80] assumed that, when the enzyme is papain, then, in contrast to serine proteases, it reacts preferentially with the aquated electron e_{aq}^- being an SH-containing enzyme. The fundamental inactivation mechanism involves damage of the active centre by one of the water radiolysis products. Under these conditions, only one active group is important. The interaction of the radical with this group leads to the inactivation of the molecule, i.e. $(k' - k_1)/k_0 = 1$.

The hypothesis that the mechanism of the inactivation of papain (Pa) reduces to damage of the active centre has been confirmed by experiments with modified papain and also by the change in G_{in} in the presence of inhibitors.[81] When cysteamine monosulfoxide, which gives rise to the mixed disulfide Pa–S–S–R, and tetrathionate, forming the Pa–S–$S_2O_3^-$ ion, were used as the modifying agents, an increase in the radiation stability of such papain derivatives was observed (Table 8).[82]

Thus, in contrast to serine proteases, the inactivation of the sulfhydryl enzyme reduces partly to the damage of the active centre, mainly via a single impact mechanism, and this is responsible for the difference between k' and k_1 and leads to the deviation of the dose dependence from the exponential function. One should also note that e_{aq}^- can pass through the protein molecule very effectively, which should hinder the application of the target theory in calculations for SH enzymes. However, by virtue of the appreciable electron affinity of the disulfide bridges, such estimates are nevertheless possible.[48] As already mentioned, the dose curves for the serine proteases, which depend on the direct damage of the serine in the active centre, also do not always correspond to an exponential relation. It is difficult to account for these findings, because the authors[78,79] did not take into account the changes in the conformation of the papain molecule, assuming this process to be of secondary importance. However, it must inevitably occur as a result of the formation under the influence of ionising radiation of S–S bonds and mixed disulfides of the type Pa–S–S–R, which alters (usually strengthens) the molecular structure and inhibits complete degradation by influencing the conformation.

The observed conformational disturbances in papain have been shown[48,87] to occur due to the decomposition of the tyrosine and tryptophan residues with preferential damage to tyrosine residues, two of which (Tyr-61 and Tyr-67) are close to the active centre. In addition, the imidazole–thiol pair (Cys-25 and His-159) plays a major role in the inactivation of papain, which affects the interaction of the enzyme with the substrate.[86] Evidently, as for trypsin-like enzymes, in the case of papain the attack by the OH radicals and the accessibility of aromatic aminoacid residues to the latter are important apart from the effect of e_{aq}^-. Table 9 presents data on the reactivities of various aminoacid residues in papain relative to OH radicals. The tyrosine and tryptophan residues head this list. The overall rate constants for the reactions of papain with OH radicals and e_{aq}^- are listed in Table 3.

Thus the additivity of two processes, namely

$$Cys + e_{aq}^- \rightarrow E_{in},$$

$$Tyr(Trp) + OH \rightarrow E_{in} + E_{ac}^*,$$

Table 8. Change in the catalytic activity of sulfhydryl proteases on exposure to X-rays (t = 0 °C, substrate—haemoglobin).

Enzyme	Irradiation conditions	Dose rate/ Gy s^{-1}	Dose/ Gy	10^5 [E] / M	D_{37}/Gy[a]	Ionic yield	No. of SH groups per molecule	G_{in}[a]	Residual activity (%)[a]	Ref.
Papain	0.067 M phosphate buffer, pH 7.4, air	0.14	65	1.35	30 (70)	–	0.5	0 (40)	–	79
	0.067 M phosphate buffer, pH 7.4, air	0.16	–	0.9	–	0.79	0.5	–	–	79
	0.067 M phosphate buffer, pH 7.4, air, in the presence of catalase	0.14	63	1.2	85	0.41	0.5	–	50	79
	0.067 M phosphate buffer, pH 7.4, N_2	0.16	–	0.9	–	0.44	–	–	–	79
	0.067 M phosphate buffer, pH 7.4, N_2, in the presence of catalase	0.16	–	0.9	–	0.37	–	–	–	79
	0.067 M phosphate buffer, pH 7.4	0.145	300	1.5	–	–	–	–	– (27)	48
	Acetate buffer, pH 6.8, N_2O	1.5×10^{-4}	–	2.0	–	–	–	1.00 (0.54)	–	79
	Acetate buffer, pH 6.9, N_2, Bu^{i}OH	1.5×10^{-4}	–	2.0	–	–	–	1.00 (0.53)	–	85
	pH 6.9	1.5×10^{-4}	–	2.0	–	–	–	0.43 (0.11)	–	85
	pH 6.9, $(CNS)_2^-$	1.5×10^{-4}	–	2.0	–	–	–	1.00 (0.31)	–	85
Papain-S–S–R	0.067 M phosphate buffer, pH 7.4	0.145	300	1.5	–	–	0.4	–	– (82)	48

[a] The value obtained in the presence of cysteine is indicated in brackets.

(E^*_{ac} are the molecules of active but damaged molecules) generates the papain inactivation pattern. The decrease in the esterase activity of papain under these conditions is associated,[48] as for serine proteases, with the degradation of Trp-177. Furthermore, the abstraction of a H atom from the SH group contributes to the inactivation:

$$OH + Pa{-}SH \rightarrow Pa{-}S^{\cdot} + H_2O\,.$$

If aromatic aminoacid residues are in the vicinity of the S–S bridge, then the probability of inactivation increases to the extent determined by the sum of the processes. As shown by the γ-radiolysis of cysteamine disulfide[48] and cystine,[83,84] the effect of ionising radiation on disulfides and thiols generates numerous products, which then enter into secondary reactions. Similar processes affect also the inactivation of SH enzymes.

It is of interest that papain exhibits a greater radiosensitivity than other SH-containing enzymes, such as alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase.[81] Its capacity for reactivation by cysteine is also higher. On the other hand, in view of the greater vulnerability of the active centre to the two reactions discussed above, papain is more radiosensitive also compared with serine proteases. However, one should take into

Table 9. The reactivity and accessibility of certain aminoacid residues in papain in relation to OH radicals.[48]

Aminoacid residue	n_{tot}	n_{exp}	$10^{-9}\,k_{(OH+AA)}$/ litre $mol^{-1}\,s^{-1}$	$n_{exp}\,k_{(OH+AA)}$	$\frac{n_{exp}\,k_{(OH+AA)}}{\Sigma\, n_{exp}\,k_{(OH+AA)}}$
Cysteine	1	1	12.3	12.3	0.04
Tryptophan	5	2.2	13.3	29.3	0.10
Tyrosine	19	13	10.1	131.3	0.47
Histidine	2	2	4.7	9.4	0.03
Phenylalanine	4	2	5.5	11.0	0.04

account the specific features and the sensitivity of the methods for the determination of catalytic activity, which, as shown in relation to chymotrypsin, are extremely important. Furthermore, in the determination of the activity of papain and in the calculation of G_{in} account must be taken of the fact that about 50% of the papain in the preparation may be in the oxidised (inactive) form, which hinders the comparison of data from different investigations if preliminary reactivation or purification has not been carried out.[85]

Experiments showed[48,80] that the radiation-induced inactivation of papain diminishes greatly following the addition of cysteine after irradiation, partly owing to the reaction

$$Pa^{\cdot} + Cys-SH \rightarrow Pa-H + Cys-S^{\cdot} .$$

The ratio G_{in} (in the presence of cysteine)/G_{in} (in the absence of cysteine) (relative yield) is presented in Figure 5. The protection by cysteine then depends on the real ratio [Pa]/[Cys]. Thus in nitrogen-purified solutions, it should be 1/(2–10). This is caused, apart from the reaction indicated above, by competing reactions with OH radicals and the faster reactions involving e_{aq}^-:

$$e_{aq}^- + R-SH \rightarrow R^{\cdot} + SH^- ,$$

$$Cys^{\cdot} + Cys-SH \rightarrow Cys-H + Cys-S^{\cdot} .$$

The species e_{aq}^- therefore cause a reversible inactivation, whereas OH radicals give rise to an irreversible inactivation.[85]

It is noteworthy that an increase in the pH of the solution increases as a rule G_{in}, although the relation between these quantities is not unambiguous.[81]

The influence of irradiation conditions on the radiation-induced inactivation of papain has been investigated. The authors[85] elucidated the effect on this process of the acetate, isobutyl alcohol, and CNS radicals. It was found that they all increase G_{in} (Table 8), evidently owing to the reaction

$$Pa-SH + R \rightarrow Pa-S^{\cdot} + RH .$$

The influence of hydrogen peroxide on the inactivation of papain has also been observed.[79,85,86] The peroxide gives rise to 60% of the reversible and 40% of the irreversible damage in papain.[85] The elimination of its influence by the addition of catalase diminishes the ionic yield, which hardly changes in air and under a nitrogen atmosphere. The dependence of the degree of inactivation on the presence of H_2O_2 can also be estimated from the increase in the radiation-induced inactivation of papain on reducing the dose rate in the absence of catalase.

Thus the principal difference between the mechanisms of the radiation-induced inactivation of the serine and sulfhydryl proteases consists in the fact that not only a disturbance of the conformation on degradation of the aromatic aminoacid residues but also the competing dominant process involving the single impact inactivation as a result of the damage to the active centre is observed in the latter case.

3. Other classes of proteases

Whereas the radiolysis of the serine and sulfhydryl proteases has been studied fairly completely, the remaining representatives of proteases are mentioned in the literature only incidentally.

The radiolysis of pepsin—an acid protease containing an asparagine residue in the active centre—has been examined.[70] It was shown that the overall characteristics of the inactivation of this enzyme are the same as for trypsin, chymotrypsin, and the subtilisins: in solution, the main reactive species is the OH radical; atomic hydrogen is extremely effective, but it makes a smaller contribution to the inactivation owing to its low yield. The similarity consists also in damage being sustained by the same radiation-sensitive aminoacid residue: Trp and Tyr, which was demonstrated by altering the degree of inactivation in the presence of Br_2^- and $(SCN)_2^-$ ions and also of *tert*-butyl alcohol. The parameters and conditions of the inactivation are presented in Table 6.

Figures 6 and 7 illustrate the inactivation of pepsin in the form of dose dependences and the changes in the pH dependence under various irradiation conditions. The pH dependence varies in the range pH 2–3. Taking into account also the changes for subtilisin[52] and the angiotensin converting enzyme (ACE) (Figure 7), one may say that the radiation-induced inactivation makes it possible to determine the conditions under which conformational changes in the molecule take place.

In the case of carboxypeptidase A, the effectiveness of the action of water radiolysis products on its enzyme activity does not differ from that for serine proteases,[69] despite the fact that the

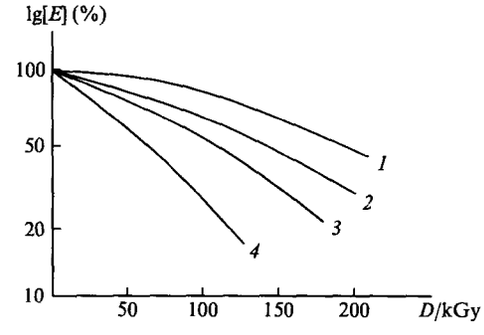


Figure 6. Dependence of the radiosensitivity of pepsin on the irradiation conditions:[69] (*1*) Ar + *tert*-BuOH; (*2*) O_2; (*3*) Ar; (*4*) N_2O.

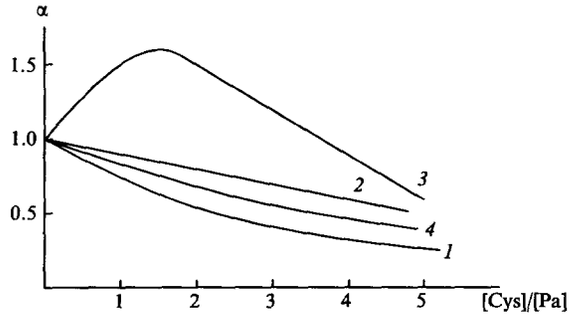


Figure 5. The relative inactivation yield (α) as a function of [Cys]/[Pa]: (*1*) calculated fraction of OH radicals reacting with papain in N_2-saturated solutions; (*2*) irreversible inactivation; (*3*) overall inactivation; (*4*) fraction corresponding to the interaction with papain of both radicals ($OH + e_{aq}^-$) in N_2-saturated solutions.

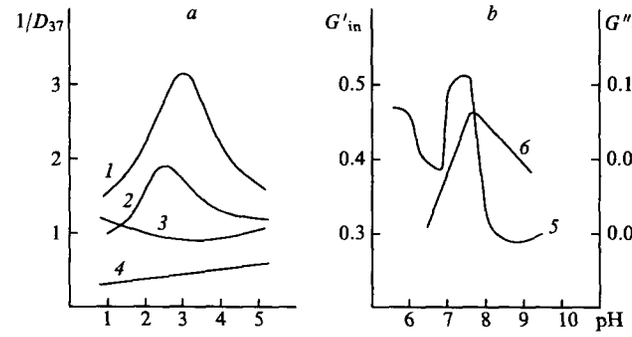


Figure 7. Variation of the radiosensitivity of pepsin[69] (*a*), subtilisin-72 [G'_{in}; curve 5 (*b*)],[52] and the angiotensin converting enzyme (ACE) [G''_{in}; curve 6 (*b*)] under different conditions: (*1*) N_2O + KBr; (*2*) N_2O; (*3*) O_2; (*4*) N_2O + KSCN.

active centre contains in this case a metal (Zn) ion. A similar radiation-chemical yield of the inactivation process is also observed (G_{in}, Table 6). All this confirmed that the inactivation of enzymes of this class takes place primarily via the alteration of the conformation and not via a single impact mechanism involving damage to the active centre. An appreciable dependence of the inactivation on the conformational state of the molecule has been observed also for a Zn-containing hydrolase—ACE, for the stability of which the number of decomposed aromatic aminoacid residues is important. Their decomposition to the extent of ~50% made the active centre accessible to radicals and caused a decrease in enzyme activity (Figure 8).

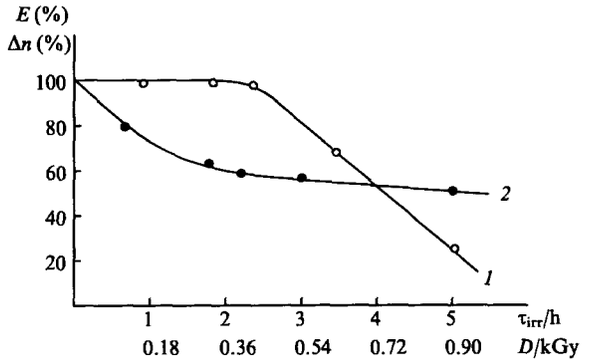


Figure 8. Variation of the catalytic activity E (curve *1*) and of the number of tryptophan and tyrosine residues (curve *2*) in the angiotensin-converting enzyme (γ-irradiation).

Overall, the radiation-induced inactivation of proteases like, incidentally, that of any other enzyme, can be represented by a scheme (Figure 9), the use of which gives rise to a greater complexity of the expressions obtained by Phihl and Sanner[79] and Gaucher et al.[80] Process *4* (Figure 9), which constitutes the mechanism of the inactivation proper, i.e. the modification of the biomolecule, depends on the coefficients of the effectiveness of the action of the water radiolysis products on the given enzyme, involving the active centre itself or its immediate environment. The highest yield and the highest effectiveness are those of the OH radicals and e_{aq}^-. The yield of H atoms, whose effectiveness is a maximum, is insufficient to influence greatly the inactivation, so that OH and e_{aq}^- actually make the main contribution to the given process. On irradiation of the pure enzyme, fundamental differences are observed between SH-containing enzymes and the remaining proteases regardless of whether their active centre contains serine, aspartic acid, or a metal atom. Apart from undergoing conformational damage due to the interaction of OH radicals with tryptophan and tyrosine residues, in the biomolecules containing a SH group the cysteine in the active centre is acted upon directly by e_{aq}^-, which increases their G_{in}. When the mechanism of the inactivation is identical, the area of the accessible surface of the molecule, characterising the extent to which it has been uncoiled, assumes fundamental importance.

Table 10. Accessible surface areas for different classes of proteases.

Enzyme	Accessible surface area/Å²	Ref.
Chymotrypsin	12996	88
Carboxypeptidase A	11260	89
Subtilisin	9790	88
Papain	9210	88

Table 10 presents certain accessible surface areas for metal-containing, SH-containing, and serine proteases. Comparison of the values of G_{in} (Table 6) shows that they are similar for chymotrypsin and carboxypeptidase. For subtilisin, which has a smaller accessible surface area, the radiation-chemical inactivation yield also diminishes. However, for papain, which has a different inactivation mechanism, a higher value of G_{in} is observed despite its smaller accessible surface area. Furthermore, the radiation stability of any protease depends on the accessibility of the aromatic aminoacid residues, especially Trp and Tyr, regardless of the stability in the active centre.

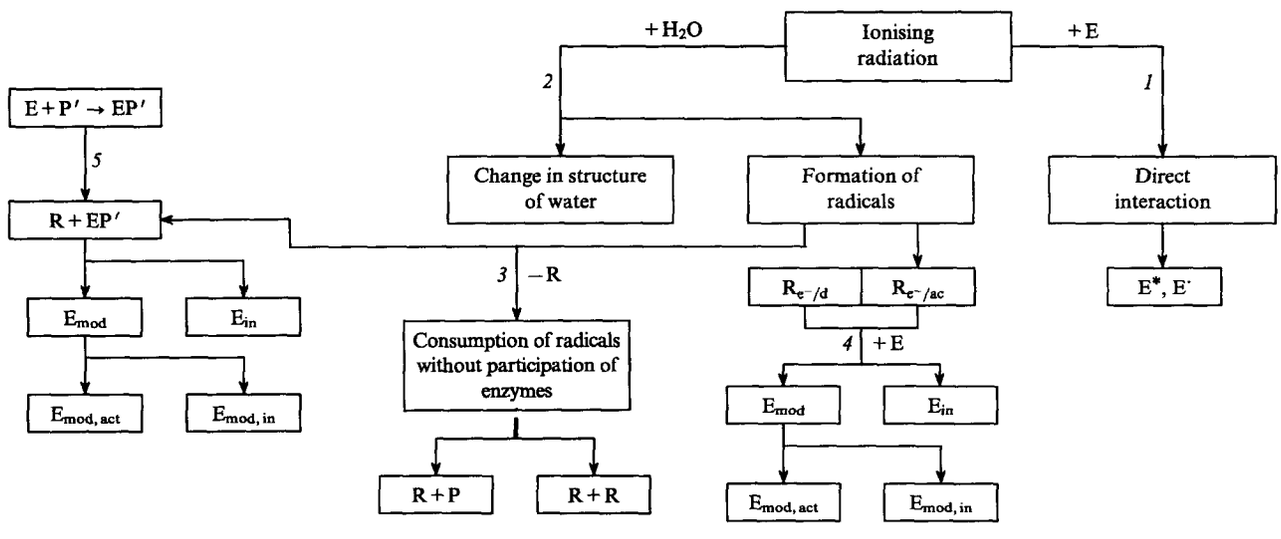


Figure 9. Schematic illustration of the interaction of enzymes with ionising radiation; E = native enzyme, E· = radical fragments of the enzyme molecule, E* = excited enzyme molecules, E_{in} = inactivated enzyme, E_{mod} = enzyme modified in any way, $E_{mod,act}$ = enzyme modified without change in activity, $E_{mod,in}$ = enzyme modified with a change in activity, R = radicals derived from water, $R_{e^-/d}$ = electron-donor radicals, $R_{e^-/ac}$ = electron-acceptor radicals, P and P′ = chemical impurity molecules present in solution.

IV. Application of the method of radiation-induced inactivation of proteolytic enzymes

There are fairly good prospects for the application of the radiation-induced inactivation method. This was partly discussed above.

The oldest and best known application of the radiation-induced inactivation method involves its use in the determination of the molecular masses of biomolecules. The possibility of characterising complex supermolecular formations is of special interest.[18] The application of the method for this purpose has been well developed and has been described in detail.[5,17,18,23,35] It is convenient, reliable, and is fairly widely used.

Medical applications of the results of the study of proteases with the aid of radiation are extremely promising. Immobilised enzymes, which, as stated above, are much more stable than the enzymes in solution, are used in this instance. An important factor is that the action of such materials on the organism is prolonged and requires very low enzyme concentrations.[41-43] Mainly the use of serine proteases, in particular trypsin, has been described. The aim of the preliminary radiation treatment is simultaneous immobilisation and sterilisation virtually without affecting the enzyme activity.

The methods for the generation of half-tone images by exposing protease-containing materials to different types of radiation give rise to extensive possibilities for the creation of dosimetric systems based on them.[40] The sensitivity of such materials can be increased (i.e. the lower limit of the doses determined can be reduced) by altering the material of the support, by introducing additives into the latter, and also by increasing the concentration of the enzymes (the latter is less desirable owing to the increase in the cost of the material and the possibility of partial radiolysis). Apart from purely dosimetric purposes, some of the systems examined[39] can serve as a material which provides an alternative to the X-ray films used in autoradiography and medicine.

In connection with the sharp impairment of the radioecological situation, various biochemical models designed to estimate the degree of risk from ionising radiation simultaneously with the risk from the usual domestic and medical factors (domestic chemistry, fertilisers, pesticides, medicines) have been widely developed in recent years.[8] In addition, another aim of these studies involves the possibility of screening the radioprotectors. In contrast to tests on animals, biochemical models are simpler and yield additional ideas concerning the mechanism of the action of the reagent under certain conditions. Among the models developed, there are also enzyme models, in particular hydrolase models.[9] An attempt was made to use enzyme models in the 1950s, but they proved to be ineffective because of the linear treatment of the results. It was found that the correlation between the effect of radiation on the organism and enzymes is more complex and is associated with the influence of effectors (activators and inhibitors), which leads to a greater or smaller imbalance in the systems of the organism under the influence of ionising radiation or other types of radiation. Figure 10 illustrates the correlation between the constant for the inhibition (K_{in}) of chymotrypsin by certain familiar radioprotectors and the dose characteristic (D_{80}) for which 80% of the enzyme remains active. The type of inhibition (competitive, noncompetitive) does not exert an appreciable influence on the above relation.[90] It must be borne in mind that any compounds used in man's daily life, including food components, medicines, perfumes, etc., may function as effectors. Thus many substances serve as radiomodifying agents, which alter the possible action of any type of radiation. The use of enzyme models may characterise the influence of the radiomodifying agent on a fully defined mechanism of the action of radiation — in particular the protease model yields information about the conformational mechanism.

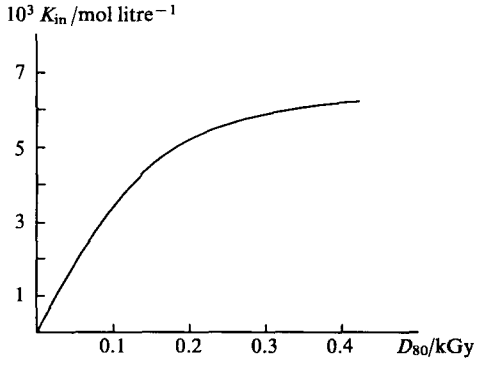


Figure 10. Dependence of the constant for the inhibition of chymotrypsin[90] by radiomodifying agents on D_{80}.

References

1. I Ya Vasilenko, Yu I Moskalev, V N Strel'tsov *Voprosy Onkologii* **31** 3 (1985)
2. N F Timofeev-Resovskii (Editor) *Pervichnye Radiobiologicheskie Protsessy* (Primary Radiobiological Processes) (Moscow: Atomizdat, 1973)
3. A V Savich (Ed.) *Radiatsionnoe Porazhenie i Vosstanovlenie Struktur i Funktsii Makromolekul* (Radiation Damage and Restoration of the Structures and Functions of Macromolecules) (Moscow: Meditsina, 1977)
4. N F Timofeev-Resovskii, A V Savich, M I Shal'nov *Vvedenie v Molekulyarnuyu Radiobiologiyu* (Introduction to Molecular Radiobiology) (Moscow: Meditsina, 1981)
5. A Swallow *Radiation Chemistry of Organic Compounds* (Translated into Russian) (Moscow: Inostr. Lit., 1963)
6. M V Vladimirova *Radiatsionnaya Khimiya Aktinoidov* (Radiation Chemistry of the Actinides) (Moscow: Energoizdat, 1983)
7. W M Garrison *Chem. Rev.* **87** 381 (1987)
8. M M Vilenchik *Radiobiologicheskie Effekty i Okruzhayushchaya Sreda* (Radiobiological Effects and the Environment) (Moscow: Energoizdat, 1983)
9. M A Orlova, S E Tkachenko, T P Trofimova, N N Troshina, V M Fedoseev, I V Golubtsov, V V Kuusk *Biochem. Biotechnol. Elect. Express Data* **1** 15 (1991)
10. J F Fowler *Phys. Med. Biol.* **29** 97 (1984)
11. L Rosenberg, M Awschalam, R K Ten Haben, B R Bennett *Health Phys.* **46** 407 (1984)
12. H Wakabayashi, Sh Suzuki, A Ite *J. Radiat. Res.* **24** 305 (1983)
13. P Andreo, A Brahme *Acta Radiol. Oncol. Rad. Ther. Phys. Biol.* **22** (1983) (Suppl. N 364. p.25)
14. L Frankenberg *Radiat. Res.* **97** 329 (1984)
15. V N Orekhovich, L A Lokshina, Yu E Eliseeva, L V Pavlikhin *Vestn. Akad. Med. Nauk SSSR* **8** 3 (1984)
16. N I Larionova, Doctoral Thesis in Chemical Sciences, Moscow State University, Moscow, 1992
17. E Pollard, A Buzzell, C Jeffrays, F Forro *Arch. Biochem. Biophys.* **33** 9 (1951)
18. J C Ellory *TJBS* 99 (1979)
19. M Houslay, J C Ellory, G Smith, T Hesketh, J Stein, G Warren, J C Metcalfe *Biochim. Biophys. Acta* **467** 208 (1977)
20. P Simon, S Swillens, J Dumont *Biochem. J.* **205** 477 (1982)
21. A S Verkman, K Skorecki, D A Ausiello *Proc. Natl. Acad. Sci. USA* **81** 150 (1984)
22. S R Levinson, J C Ellory *Biochem. J.* **137** 123 (1974)
23. K G Tsimmer *Problemy Kolichestvennoi Radiobiologii* (Problems of Quantitative Radiobiology) (Moscow: Gosatomizdat, 1962)
24. D P Alexander, D A Charlesby *Nature (London)* **173** 578 (1954)
25. R B Setlow *Proc. Natl. Acad. Sci. USA* **38** 166 (1952)
26. R B Setlow, B Doyle *Arch. Biochem. Biophys.* **46** 46 (1953)
27. A Norman, W Ginoza *Radiat. Res.* **7** 440 (1957)
28. R L Kincald, E Kempner, V C Manganielle, G C Osborne, M Vaughan *J. Biol. Chem.* **256** 11351 (1981)
29. T B Nielsen, P M Lad, M S Preston, E Kempner, W Schlegel, M Rodbell *Proc. Natl. Acad. Sci. USA* **78** 722 (1981)

30. E Kempner, W Schlegel, J H Miller, J Z Hearon *J. Biol. Chem.* **255** 6826 (1980)
31. E Kempner, W Schlegel *Anal. Biochem.* **92** 2 (1979)
32. A K Valeev, O V Malinovskii *Radiobiologiya* **23** 624 (1983)
33. M A Orlova, I I Nikol'skaya, I V Golubtsov *Radiobiologiya* **27** 308 (1987)
34. R A Deering *Radiat. Res.* **5** 238 (1956)
35. E S Pollard, W R Guld, F Hutchinson, R B Setlow *Prog. Biophys.* **5** 238 (1955)
36. T K Hopkins, J D Spikes *Radiat. Res.* **37** 253 (1969)
37. P Riesz, F H White *Radiat. Res.* **44** 24 (1970)
38. F H White, P Riesz, H Kon *Radiat. Res.* **32** 744 (1962)
39. E V Lukasheva, N F Kazanskaya, I V Berezin *Vestn. Mosk. Univ., Ser. 2: Khim.* **27** 86 (1986)
40. M A Orlova, I I Nikolskaya, I V Golubtsov, N F Kazanskaya *Radiat. Eff. Lett.* **85** 143 (1984)
41. R V Virnik, V V Ryl'tsev, L G Vlasov, E V Dovbii, A T Kalashnik *Prikl. Biokhim. Mikrobiol.* **19** 533 (1983)
42. V V Ryl'tsev, L G Vlasov, T I Samoilova, L I Volkovinskaya, L N Bondareva, V D Zazhirei, S V Netrebenko, V I Khachiyants *Prikl. Biokhim. Mikrobiol.* **20** 694 (1984)
43. R V Virnik, V V Ryl'tsev, L G Vlasov, G I Samoilova, E V Dovbii, A T Kalashnik *Prikl. Biokhim. Mikrobiol.* **21** 106 (1985)
44. L Schachinger, Ch Schippel, E Altmann, B Diepold, Ch Yang, M Jaenke *Radiat. Environ. Biophys.* **24** 259 (1985)
45. G E Adams, K F Baverstock, R B Cundall, J L Redpath *Radiat. Res.* **54** 375 (1973)
46. K R Lynn, G Orpen *Int. J. Radiat. Biol.* **14** 363 (1968)
47. J R Clement, D A Armstrong, N A Gillis *Can. J. Chem.* **50** 2833 (1972)
48. J R Clement, D A Armstrong, H N V Klassen *Can. J. Chem.* **51** 2443 (1973)
49. A G Bezrukova, J Yu Ostashevsky *Int. J. Radiat. Biol.* **31** 131 (1977)
50. K R Lynn, G Orpen *Radiat. Res.* **39** 15 (1969)
51. I I Nikol'skaya, M A Orlova, I V Golubtsov *Radiobiologiya* **26** 302 (1986)
52. I I Nikol'skaya, M A Orlova, E V Bogaevskaya *Radiobiologiya* **26** 477 (1986)
53. K Outsuki, M Fukuhara, K Sumizu, H Hatano *J. Radiat. Res.* **11** 113 (1970)
54. K Outsuki *J. Radiat. Res.* **11** 120 (1970)
55. C E Stauffer, D Etson *J. Biol. Chem.* **244** 5333 (1969)
56. R A Alden, C S Wright, F C Westall, J Kraut *Subtilisin BPN': Tertiary Structure and Inhibitor Binding* (1970)
57. L K Mee *Radiat. Res.* **21** 501 (1964)
58. L K Mee *Radiat. Res.* **9** 151 (1958)
59. T W Smith, S J Adelstein *Radiat. Res.* **19** 213 (1963)
60. L A Mounter *Radiat. Res.* **12** 487 (1960)
61. E C Moore, M McDonald *Radiat. Res.* **3** 38 (1955)
62. M McDonald *J. Gen. Physiol.* **38** 581 (1955)
63. M McDonald *J. Gen. Physiol.* **38** 93 (1955)
64. M McDonald *Br. J. Radiol.* **27** 62 (1954)
65. K R Lynn *Radiat. Res.* **46** 268 (1971)
66. A G Bezrukova, I Yu Ostashevsky *Int. J. Radiat. Biol.* **31** 349 (1977)
67. M A Orlova, I V Golubtsov, I I Nikol'skaya *Vestn. Mosk. Univ., Ser. 2: Khim.* **28** 601 (1987)
68. M A Orlova, I I Nikol'skaya, E V Bogaevskaya, I V Golubtsov *Radiobiologiya* **26** 844 (1986)
69. PB Roberts *Int. J. Radiat. Biol.* **24** 143 (1973)
70. G E Adams, M I Pesener, R H Bisby, R B Cundall, J R Key *Int. J. Radiat. Biol.* **35** 497 (1979)
71. D Aronson, L Mee, C L Smith, in *Progress in Radiobiology, Proceedings of IV Internatinal Conference on Radiobiology, Cambridge, 1955* p. 61
72. K R Lynn *Radiat. Res.* **43** 525 (1970)
73. V V Mosolov *Proteoliticheskie Fermenty* (Proteolytic Enzymes) (Moscow: Nauka, 1971)
74. T Masuda, J Ovadia, L L Grossweiner *Int. J. Radiat. Biol.* **20** 447 (1971)
75. Ch Ts'ao, W F Ward *Radiat. Res.* **101** 394 (1985)
76. Ch Ts'ao, W F Ward, C D Port *Radiat. Res.* **96** 301 (1983)
77. E Ya Graevskii *Sul'fgidril'nye Gruppy i Radiochuvstvitel'nost'* (The Sulfhydryl Groups and Radiosensitivity) (Moscow: Atomizdat, 1969)
78. T Sanner, A Phihl *Radiat. Res.* **19** 12 (1963)
79. A Phihl, T Sanner *Radiat. Res.* **19** 27 (1963)
80. G M Gaucher, B L Mainman, G P Thompson, D A Armstrong *Radiat. Res.* **46** 457 (1971)
81. S Fuimoto, T Nakagawa, S Ishimitsu, A Ohara *Chem. Pharm. Bull.* **31** 992 (1983)
82. J W Purdie *Can. J. Chem.* **49** 725 (1971)
83. J W Purdie *J. Am. Chem. Soc.* **89** 226 (1967)
84. D W Grant, S N Mason, M A Link *Nature (London)* **192** 352 (1961)
85. G M Gaucher, D A Armstrong *Can. J. Chem.* **51** 2443 (1973)
86. W S Lin, G Maurice, M Lal, D A Armstrong *Can. J. Chem.* **54** 242 (1976)
87. G Beguta, A M Dancewicz *Int. J. Radiat. Biol.* **43** 249 (1983)
88. C Chetskia *Nature (London)* **254** 304 (1975)
89. C Chetskia *J. Mol. Biol.* **105** 1 (1976)
90. M A Orlova, S E Tkachenko, T P Trofimova, N N Troshina, V M Fedoseev *Vestn. Mosk. Univ., Ser. 2: Khim.* **34** (1993) (in the press)