THE INACTIVATION OF DILUTE SOLUTIONS OF CRYSTALLINE TRYPSIN BY X-RADIATION

II. Effects of Enzyme Concentration, Medium, pH, and Temperature*

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Previous studies in this laboratory (1) on the inactivation of dilute solutions of crystalline trypsin in 0.005 N hydrochloric acid by x-radiation at room temperature showed that the reaction yield, that is, the number of micromoles of trypsin per liter inactivated per 1000 roentgens (r), increases markedly as the initial concentration of trypsin is increased from 0.1 to 200 micromolar (μM). On the other hand, Dale and his coworkers (2) demonstrated that the reaction yield for the inactivation of aqueous solutions of crystalline carboxypeptidase by x-rays is constant for concentrations of enzyme between 6 and 600 μM. Only a slight rise in the reaction yield for the inactivation of aqueous solutions of crystalline pepsin by high voltage electrons was observed by Bellamy and Lawton (3) when the concentration of pepsin was increased from 2.8 to 140 μM. These are the only pure enzymes that have been studied extensively over a wide concentration range.

Fricke's observations (4) on the reactivity of hydrochloric acid in radiation studies raised the question whether the continuous rise in reaction yield with increasing concentration of trypsin obtained in the previous studies (1) was due to the presence of hydrochloric acid in the solutions. A detailed study was therefore made of the inactivation by x-radiation of dilute solutions of trypsin in diverse media under various experimental conditions. This showed that the chemical nature of the medium in which the trypsin is irradiated markedly affects the reaction yield. When solutions of trypsin of various concentrations in 0.005 N hydrochloric, sulfuric, or nitric acid were irradiated, the reaction yields were found to increase as the concentration of enzyme was increased. The rise was less pronounced in the presence of sulfuric or nitric acid, however, than in the presence of hydrochloric acid. With concentrations of trypsin between 1 and 10 μM, the reaction yields obtained in 0.005 N hydrochloric acid

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were much lower than those obtained in 0.005 N sulfuric or nitric acid, but no significant differences were observed with 100 μM solutions of trypsin. The rate of inactivation was found also to be a function of pH, minimum yields being obtained at approximately pH 7. The differences between the reaction yields obtained in the presence of sulfate and chloride ions were found to be significant only in the pH range from 2 to 4. When solutions of trypsin were irradiated at 26 and at 5°C., larger reaction yields were found at the higher temperature, with 0.005 N hydrochloric acid as the solvent. The dependence on temperature was less when 0.005 N sulfuric acid was used, and negligible with 0.005 N nitric acid. Many compounds, such as acetate buffer, glucose, and ethanol, were found to inhibit the inactivation. Irradiation in the presence of ethanol produced sigmoid inactivation-dose curves instead of the customary exponential ones.

Materials and Methods

All the materials and procedures used in this study were the same as those described in the first paper of the series (1) with the following exceptions. The trypsin was dialyzed against distilled water instead of 0.005 N hydrochloric acid before lyophilization. The tubes containing the samples to be irradiated were, unless otherwise stated, immersed in crushed ice during the irradiation process (temperature of solu tions, 2–5°C.) and stoppered with 1.5 mm. aluminum caps. The dosage rate was about 300 r per minute.

Unless otherwise specified, each reaction yield was calculated from data from nine aliquots irradiated for different time intervals, spaced to give from 10 to 90 per cent inactivation, by means of the following equation. The calculated reaction yields are for the initial period of the reaction, since the inactivation of trypsin solutions by x-rays is a reaction of the first order, the rate of inactivation at any given instant during irradiation being directly proportional to the concentration of trypsin at that instant (1, 10, 11).

$$Y = \frac{2.3TB}{MSN} \times 10^9$$

in which Y = reaction yield = number of micromoles of trypsin per liter initially inactivated per 1000 roentgens,

T = number of trypsin units per milliliter present before irradiation,

B = slope of the regression line[1] (calculated by the method of least squares) resulting when the common logarithms of the number of trypsin units per milliliter are plotted against the radiation dose,

M = molecular weight of trypsin,[2]

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[1] The term regression line is employed in statistical terminology to designate the straight line used to estimate one variable from another.

[2] The value used in these calculations was 36,500 (5). Should the more recent estimates of the molecular weight of trypsin (6–8), indicating values of 15,000 to 21,000,

S = specific activity of the trypsin sample in units per milligram of nitrogen,
N = nitrogen content of the trypsin sample in per cent,
2.3 = factor for the conversion of common to natural logarithms.

RESULTS

Relation of Reaction Yield to Initial Trypsin Concentration: Effect of Various Anions.—Inactivation curves were obtained at 2–5°C. for solutions of trypsin ranging from 1 to 300 μM in 0.005 N hydrochloric, sulfuric, or nitric acid, and the reaction yields were calculated.[3] The data are presented in Table I. It is evident that the reaction yields for the inactivation of solutions of trypsin by x-rays increase as the initial concentration of trypsin is increased,[4] regardless of whether 0.005 N hydrochloric, sulfuric, or nitric acid is used as the solvent. It is also evident that the increase is much greater in the case of hydrochloric acid than in the case of either sulfuric or nitric acid. The marked differences in the reaction yields observed for the three solvents with 1 to 10 μM trypsin solutions are not evident with 100 μM solutions.

It was shown previously (1) with regard to the inactivation by x-rays of dilute solutions of trypsin in 0.005 N hydrochloric acid at room temperature that, when the reaction yields are plotted as a function of the initial concentration of trypsin, they fall approximately on a curve given by the expression $Y \alpha X^K$, in which Y is the reaction yield, X is the concentration of trypsin, and K is a constant. Similar results are obtained when trypsin solutions are irradiated at 2–5°C. in 0.005 N hydrochloric, sulfuric, or nitric acid. This is illustrated in Fig. 1, where it can be seen that there is a straight line relationship between the logarithms of the initial concentrations of trypsin and the logarithms of the reaction yields. The calculated values for K (slope of the regression line) were found to be 0.464 (0.445–0.482),[5] 0.203 (0.181–0.225), and 0.158 (0.137–0.179) when hydrochloric, sulfuric, and nitric acids, respectively, were used. The

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prove to be correct, the reaction yields and enzyme concentrations given previously (1) and in this paper will be approximately half the correct values. It should be noted, however, that the molecular weight of trypsin as determined by deuteron and electron bombardment (9) closely approximates that used here.

[3] Since the maximum time of irradiation was 3 hours, complete inactivation curves (10 to 90 per cent inactivation) were obtained only with the lower concentrations of enzyme. With concentrations of trypsin greater than 3, 8, and 13 μM, respectively, and with hydrochloric, sulfuric, and nitric acids as the solvents, the maximum inactivation obtained ranged from 90 to 20 per cent.

[4] Whether the reaction yields would continue to increase with concentrations of trypsin greater than 300 μM could not be ascertained with our equipment.

[5] Mean and 95 per cent fiducial limits, the latter calculated as the mean, plus or minus the product of the standard error and the critical value of t at the 5 per cent probability level for the degrees of freedom involved.

TABLE I

Variation in Reaction Yield with Variation in Initial Trypsin Concentration: Effect of Anions

Solvent*	0.005 N HCl	0.005 N H_2SO_4	0.005 N HNO_3
Initial trypsin concentration	Reaction yield		
micromoles per liter	*micromoles trypsin per liter inactivated per 1000 r*‡		
0.941		0.277 (0.267–0.288)	0.312 (0.305–0.319)
1.07	0.065 (0.061–0.069)		
1.18	0.074 (0.066–0.082)		
1.38		0.306 (0.301–0.311)	0.363 (0.348–0.379)
1.63	0.084 (0.080–0.089)		
2.61		0.344 (0.304–0.384)	0.399 (0.384–0.415)
2.68	0.108 (0.104–0.112)	0.337 (0.328–0.346)	
2.82	0.101 (0.088–0.115)		
3.08	0.113 (0.101–0.124)		
3.31			0.404 (0.388–0.420)
4.24			0.399 (0.386–0.412)
4.64	0.149 (0.130–0.167)	0.362 (0.347–0.376)	
7.57	0.175 (0.162–0.189)		
7.95		0.413 (0.399–0.427)	
8.85			0.525 (0.460–0.590)
12.1		0.429 (0.420–0.438)	0.578 (0.548–0.609)
12.4			0.535 (0.520–0.551)
14.0	0.269 (0.252–0.286)		
18.3		0.444 (0.421–0.466)	0.580 (0.566–0.594)
19.2	0.270 (0.253–0.286)	0.477 (0.448–0.505)	0.572 (0.535–0.609)
28.5			0.540 (0.520–0.560)
31.4	0.325 (0.291–0.360)		0.614 (0.593–0.635)
32.7	0.336 (0.310–0.363)		
50.3	0.435 (0.385–0.485)	0.643 (0.596–0.691)	0.572 (0.530–0.615)
53.8			0.645 (0.606–0.683)
76.3	0.512 (0.450–0.575)	0.652 (0.593–0.711)	
76.8		0.675 (0.652–0.699)	0.655 (0.614–0.695)
79.6			0.726 (0.662–0.790)
98.9		0.634 (0.523–0.745)	
105			0.764 (0.668–0.860)
107	0.664 (0.605–0.723)	0.653 (0.551–0.756)	0.681 (0.553–0.810)
114	0.644 (0.556–0.731)		
254		0.813 (0.642–0.984)	
262	1.00 (0.772–1.23)		0.718 (0.561–0.874)
265	0.850 (0.631–1.07)	1.03 (0.850–1.20)	0.881 (0.678–1.08)
273	0.831 (0.602–1.06)		
275	0.803 (0.670–0.936)		

* pH of solutions, 2.4–2.6.

‡ Mean and 95 per cent fiducial limits.

reasons for the differences in slope are obscure. A similar relationship between reaction yield and solute concentration has been observed in studies on the deamination of glycine[6] (12, 13) and L-serine (14), and the liberation of hydrogen sulfide from cysteine hydrochloride and glutathione (15) by x-rays. Possible reasons for a reaction yield–solute concentration relationship of this type have been recently reviewed by Dale (16).

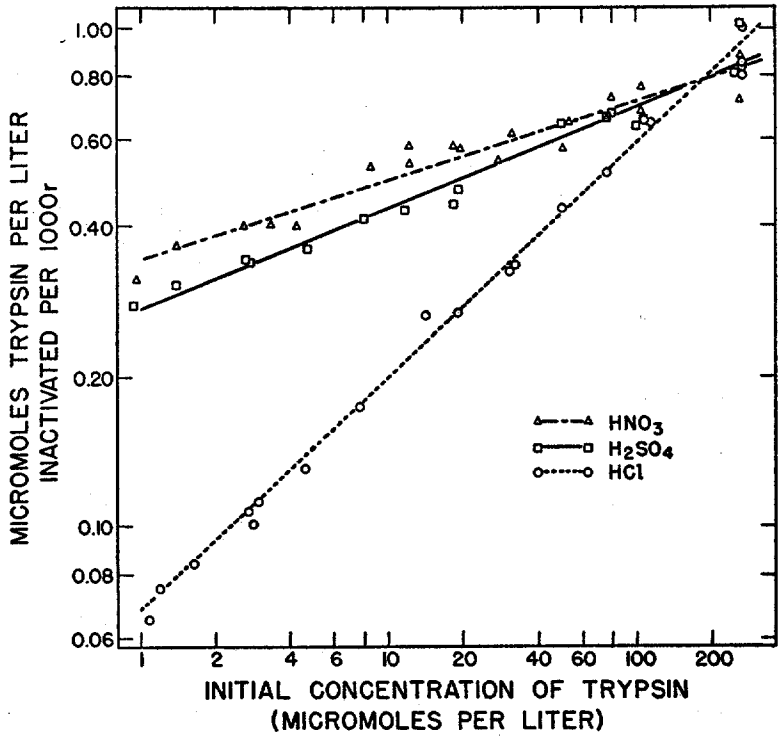

FIG. 1. Variation in reaction yield with variation in initial trypsin concentration. The slope of the regression line furnishes K, the constant in the theoretical equation given in text.

Effect of pH on the Reaction Yield.—When 0.005 N sodium (or potassium) chloride, sulfate, and nitrate were substituted for the respective acids as the solvents for the trypsin solutions in the x-ray inactivation studies, it was noted that the differences between the reaction yields observed with the three anions were much less pronounced than when the acids were used. This is illustrated in Table II. It should be noted that whereas the yields in 0.005 N hydrochloric acid were lower than those in water, as, to a much

[6] K was found in this case to be 0.3 (12).

smaller extent, were the yields in the 0.005 N salt solutions, the yields in 0.005 N sulfuric and nitric acids were greater. Similarly, when the concentration of the electrolytes was raised to 0.1 N, the differences between the yields observed with the chloride and sulfate ions became negligible, although there was a twofold difference between the acids and the salts.

The study of the true effect of pH on the inactivation of trypsin by x-rays is complicated by the inherent fact that it is impossible to vary the hydrogen ion

TABLE II

Effect of Various Electrolytes on the Reaction Yield for the Inactivation of Trypsin Solutions by X-Radiation

Solvent	pH of solution*	Initial trypsin concentration	Reaction yield‡
		micromoles per liter	*micromoles trypsin per liter inactivated per 1000 r*
H_2O	5.1	2.43	0.268 (0.252–0.285)
0.005 N NaCl	4.9	2.53	0.198 (0.184–0.213)
0.005 N KCl	5.0	2.66	0.202 (0.196–0.208)
0.005 N Na_2SO_4	5.1	2.31	0.231 (0.213–0.250)
0.005 N $NaNO_3$	5.0	2.42	0.223 (0.217–0.229)
0.005 N HCl§	2.5	2.48	0.104 (0.098–0.110)
0.005 N H_2SO_4§	2.5	2.48	0.328 (0.305–0.352)
0.005 N HNO_3§	2.4	2.48	0.396 (0.369–0.424)
0.1 N NaCl	4.8	2.55	0.211 (0.192–0.230)
0.1 N Na_2SO_4	5.2	2.57	0.215 (0.200–0.231)
0.1 N HCl	1.6	2.59	0.437 (0.405–0.469)
0.1 N H_2SO_4	1.8	2.52	0.449 (0.418–0.479)

* No significant change in the pH of the solutions was observed during the irradiation process.

‡ Mean and 95 per cent fiducial limits.

§ Values calculated from the data of Fig. 1.

concentration of the solutions without changing the chemical nature of the medium, which may also affect the reaction. All the buffer systems tried as solvents were found to inhibit the inactivation to some extent (Table III). This is true even of simple inorganic salts (*cf.* Table II). Two systems were finally selected as the least objectionable. The first was an unbuffered one, composed of mixtures of sodium sulfate and sulfuric acid or sodium hydroxide in which the concentration of sodium and sulfate ions was kept essentially constant; the second was Teorell and Stenhagen's universal buffer (an alkaline salt mixture of sodium citrate, phosphate, and borate to which are added

various amounts of hydrochloric acid). Although the latter greatly inhibits the amount of inactivation obtained with any given dose of radiation, the concentrations of inhibiting constituents do not vary with the pH of the buffer. Since marked differences had been observed in the reaction yields obtained in the presence of sulfate and chloride ions at pH 2.5 but not at pH 5.0 or 1.7 (Table

TABLE III

Effect of Various Buffers on the Inactivation of Trypsin Solutions by X-Rays

Initial concentration of trypsin, 1 μM; dose, 10,000 r.

Solvent	Amount of trypsin inactivated*
	per cent
Water‡	86
0.001 M Sørensen's phosphate buffer (17), pH 6.1	83
0.01 M " " " "	80
0.1 M " " " "	69
Water‡	80
0.0025 M Walpole's acetate buffer (18), pH 4.1	50
0.01 M " " " "	31
0.05 M " " " "	15
Water‡	83
Teorell and Stenhagen's universal buffer (19), pH 5.0§	16
Water‡	83
0.001 M‖ Michaelis' universal buffer (20), pH 5.3	5
0.005 M " " " "	4
0.025 M " " " "	1

* Reaction yields were not calculated in these experiments since complete inactivation curves were not run, and it is not known whether true exponential curves would have been obtained in all cases.

‡ pH of water solutions adjusted with dilute sulfuric acid or sodium hydroxide to that of the buffer solution.

§ Composition, 0.0095 M phosphoric acid, 0.0108 M boric acid, 0.0064 M citric acid, 0.066 M sodium hydroxide, and 0.022 M sulfuric acid.

‖ Molarity calculated on basis of diethyl barbiturate concentration. Composition, 0.001 M hydrochloric acid, 0.001 M sodium acetate, 0.001 M sodium diethyl barbiturate, and 0.04 M sodium chloride.

II), analogous systems of sodium chloride and hydrochloric acid or sodium hydroxide, and Teorell and Stenhagen's buffer with sulfuric acid substituted for the hydrochloric acid constituent, were also used to determine the pH range in which chloride ions apparently inhibited the reaction.

Data showing the effect of pH on the reaction yields, using mixtures of 0.1 N sodium chloride and 0.1 N hydrochloric acid or sodium hydroxide, or mixtures

of 0.1 N sodium sulfate and 0.1 N sulfuric acid or sodium hydroxide as the solvents for the trypsin solutions, are presented in Fig. 2. It is evident that the differences in the reaction yields found with sulfate and with chloride ions are significant only in the pH range from 2 to 4, with the maximum difference at approximately 2.5, the pH at which the data in Table I and Fig. 1 were collected. The reaction yield–pH curve obtained in the presence of sulfate ions is a rather symmetrical one, with minimum yields at approximately pH 7. The

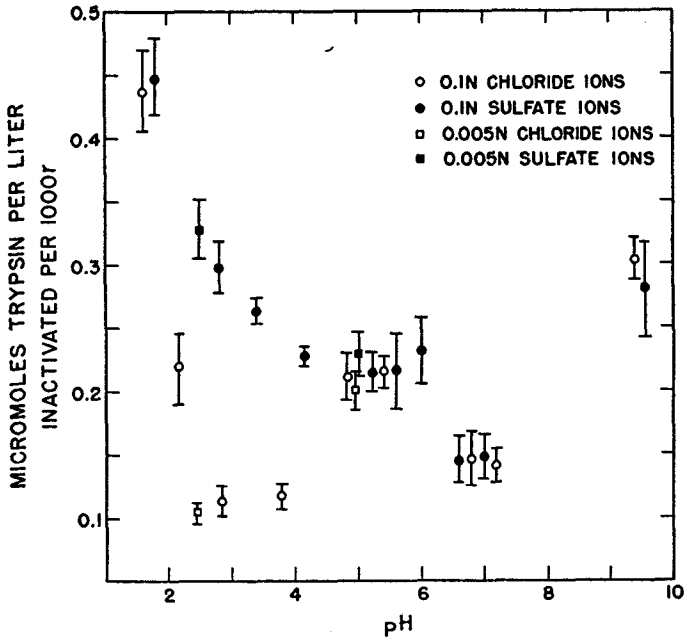

FIG. 2. Effect of pH on the inactivation of trypsin solutions by x-radiation in the presence of sulfate or chloride ions. Concentration of trypsin solutions, 2.5 μM. Maximum time of irradiation, 60 minutes. Points represent the mean, vertical lines, the 95 per cent fiducial limits.

curve obtained in the presence of chloride ions, however, shows two distinct minima at approximately pH 3 and pH 7. Four of the points in Fig. 2 were obtained with 0.005 N solutions of electrolytes as solvents—two at pH 2.5 with hydrochloric acid and sulfuric acid, one at pH 4.9 with sodium chloride, and one at pH 5.0 with sodium sulfate. These points fall on the curve obtained with 0.1 N solutions of the electrolytes as solvents, indicating that if these electrolytes exert an influence on the reaction (as the data in Table II might suggest), the maximum effect has already been reached at the lower concentrations.

The effect of pH on the reaction yields for the inactivation of dilute trypsin solutions by x-rays in the presence of Teorell and Stenhagen's buffer is illus-

trated in Fig. 3. The data are in essential agreement with those obtained when the pH was varied by using mixtures of 0.1 N sodium sulfate and 0.1 N sulfuric acid or sodium hydroxide (Fig. 2), and with the results of Bier and Nord[7] on the inactivation of trypsin solutions by high intensity electron bombardment (21), using acetate buffer as the solvent.[8] These results are in marked contrast

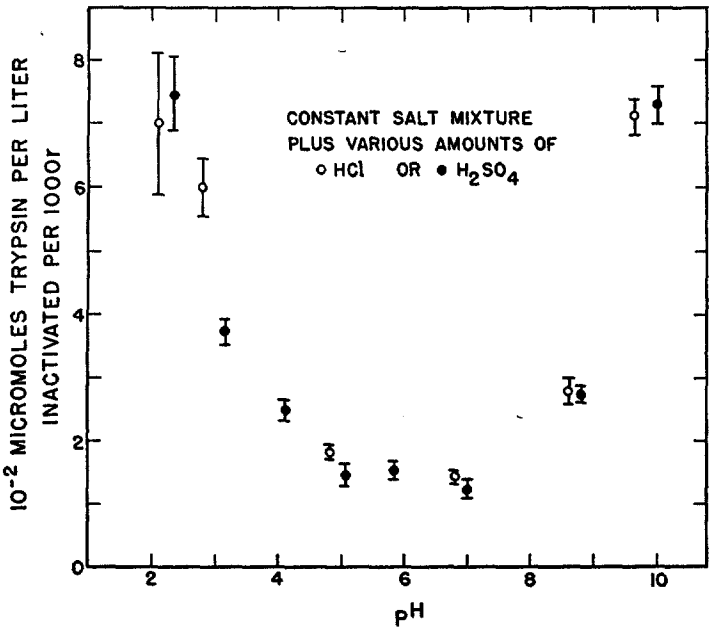

FIG. 3. Effect of pH on the inactivation of trypsin solutions by x-radiation with Teorell and Stenhagen's universal buffers as solvents. Concentration of trypsin solutions, 1 μM. Maximum time of irradiation, 120 minutes. Composition of buffer, 0.0095 M phosphoric acid, 0.0108 M boric acid, 0.0064 M citric acid, 0.066 M sodium hydroxide, plus varying amounts of hydrochloric or sulfuric acid (0.02 N at pH 10 to 0.07 N at pH 2). Circles represent the mean, vertical lines, the 95 per cent fiducial limits.

to those found in studies of the thermal inactivation of trypsin, in which the pH of maximum stability was shown to be approximately 2.6 (23).

Effect of Ethanol on the Inactivation of Solutions of Trypsin by X-Rays.—The

[7] These authors also noted that acetate buffer inhibited the inactivation of trypsin solutions by ionizing radiation.

[8] It should be noted that Barron *et al.* (22) found no differences in the reaction yields for the inactivation of trypsin by x-rays at pH 7.5 and 9.1. Their results are not directly comparable, however, since different buffers were used as solvents for the two pH values—0.05 M phosphate buffer for pH 7.5, and 0.01 M bicarbonate-carbonate buffer for pH 9.1.

"protection" effect resulting from competition of two or more solutes in one solution for the radicals produced from water by ionizing radiation is a characteristic feature of the indirect action of radiation (24–26). Examples of this phenomenon have already been noted in this paper in the reduced yields observed when trypsin solutions were irradiated in the presence of various in-

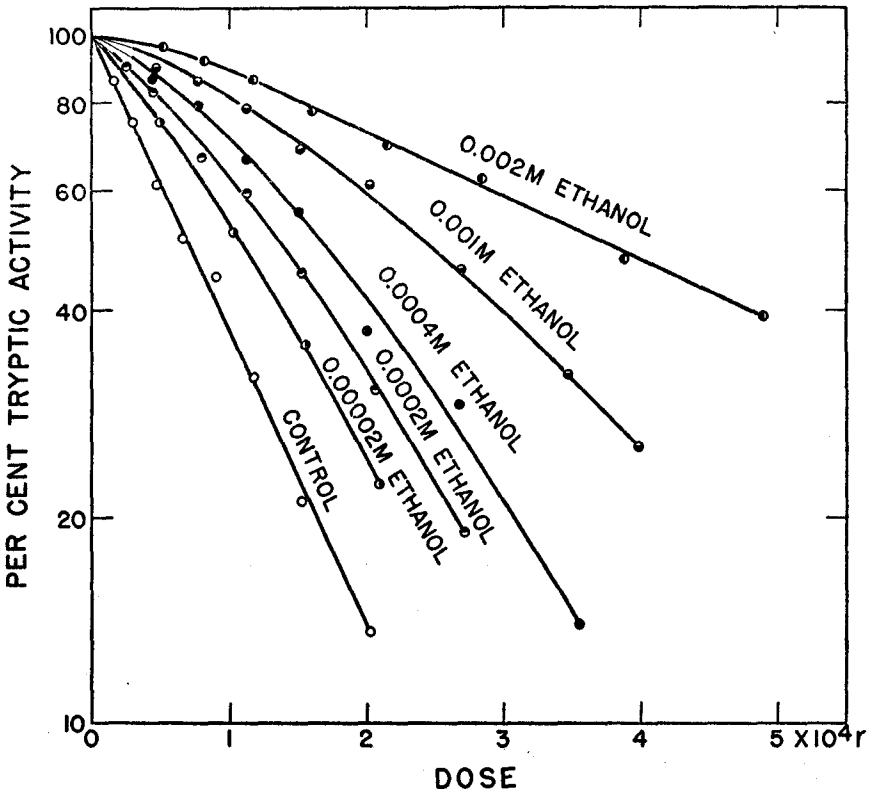

FIG. 4. Effect of ethanol on the inactivation of trypsin solutions by x-rays. Concentration of trypsin solutions, 1 μM. Solvents, 0.005 N hydrochloric acid containing various amounts of ethanol to give the concentrations indicated on chart.

organic and organic compounds. One of the more interesting examples of protection is that given by ethanol. When solutions of trypsin are irradiated in the presence of ethanol, the percentage of enzyme inactivated by any given dose of x-rays is markedly less than the percentage inactivated in the absence of ethanol. Furthermore, the inactivation-dose curves obtained are not exponential. This is shown in Fig. 4. Not all protective agents alter the shape of the inactivation-dose curves. Exponential curves are found, for example, when trypsin is irradiated in the presence of acetate buffer or Teorell and Sten-

hagen's universal buffer (Fig. 5). On the other hand, there is a break in the inactivation-dose curve when trypsin is irradiated in the presence of glucose (Fig. 5).

Effect of Temperature on the Inactivation of Trypsin Solutions by X-Rays.— Comparsion (27) of the reaction yields for solutions of trypsin in 0.005 N

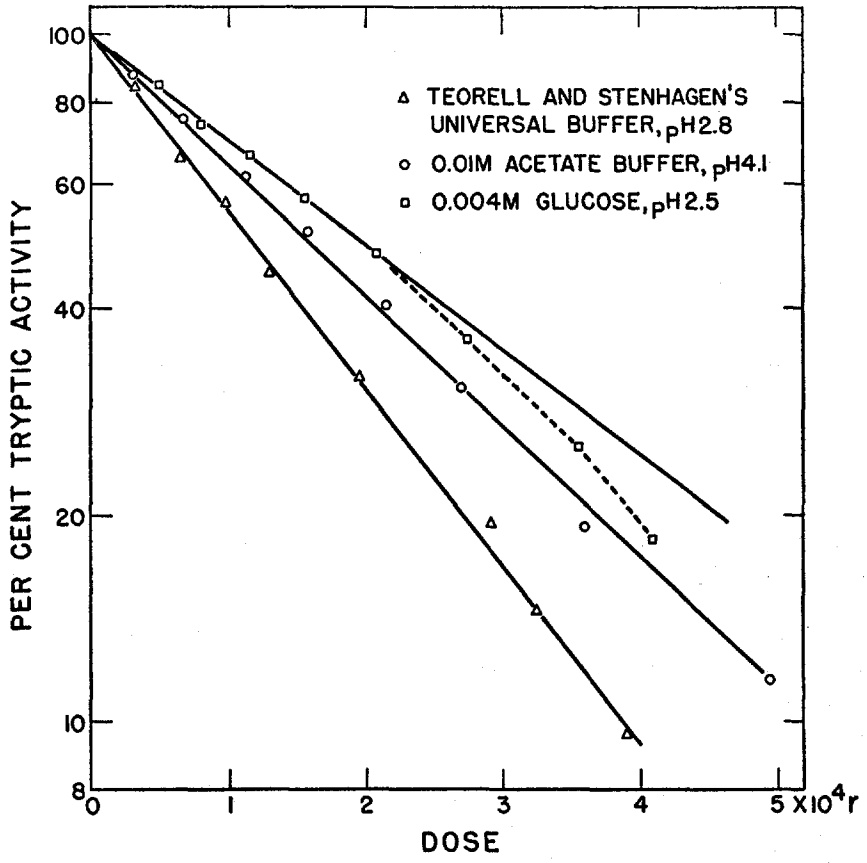

FIG. 5. Effect of various protective agents on the type of inactivation-dose curve obtained upon irradiation of trypsin solutions. Concentration of trypsin solutions, 1 μM.

hydrochloric acid, irradiated at 3°C. (Table I) and at 22°C. (1), indicated that the yields were slightly higher in the latter case. Whether temperature actually influences the reaction could not be determined precisely from these data, however, since they were procured with different trypsin preparations.[9] Results of experiments in which the same sample of trypsin was studied at

[9] When the trypsin samples involved were later irradiated under identical conditions, no significant differences were found.

different temperatures are summarized in Table IV. It is apparent that the reaction yields for trypsin solutions in 0.005 N hydrochloric acid are significantly higher when the irradiation is carried out at the higher temperature. The dependence on temperature is less when 0.005 N sulfuric acid is used, and negligible with 0.005 N nitric acid.

TABLE IV

Effect of Temperature on the Inactivation of Trypsin Solutions by X-Rays

Solvent	Temperature	Initial trypsin concentration	Reaction yield*
	°C.	*micromoles per liter*	*micromoles trypsin per liter inactivated per 1000 r*
0.005 N HCl	26	2.96	0.164 (0.150–0.178)
0.005 N HCl	5	2.96	0.107 (0.096–0.119)
0.005 N H_2SO_4	24	2.97	0.386 (0.364–0.407)
0.005 N H_2SO_4	5	2.79	0.335 (0.310–0.360)
0.005 N HNO_3	22	3.31	0.400 (0.383–0.416)
0.005 N HNO_3	5	3.31	0.404 (0.388–0.420)

* Mean and 95 per cent fiducial limits.

DISCUSSION

Many enzymes have been shown to be inactivated by ionizing radiation, both in the dry state and in solution (28). In the former case any primary ionization occurring within any part of the protein molecule appears to be sufficient to cause total loss of enzymatic activity, the radiation-sensitive volume corresponding closely to the molecular volume of the enzyme (9, 29, 30). In the latter case, the inactivation is apparently due to reaction with the radiation products of water. The composition of the medium is of great importance here since the addition of other solutes will result in competition for these radiation products, effectively lowering the number available for reaction with the enzyme (24–26). The significance of this protective effect, although amply demonstrated in many studies, has not been sufficiently appreciated by many radiation experimenters, particularly with respect to the precautions that must be taken to avoid unintentional addition of substances other than the one being studied. For example, during the course of these investigations it was noted on many occasions that much less inactivation of the trypsin solutions resulted from a given dose of radiation than was to be expected from previous experiments. These variant results were finally traced to protection of the trypsin by the inadvertent addition of other chemicals capable of reacting with "activated" water. For example irradiation of trypsin solutions that had stood for 24 hours in lusteroid tubes, or had been prepared with solvent that

had stood for 24 hours in lusteroid tubes, produced less than half the amount of inactivation resulting from irradiation of control solutions stored for the same period of time under similar conditions in glass or quartz tubes. Similar decreases were noted if the trypsin preparations were stirred before irradiation with a tygon-coated magnetic bar, whereas no such decrease resulted from stirring with a glass-enclosed magnetic bar. It is apparent that some substance or substances, probably the plasticizer, can be extracted from chemically "inert" plastics in quantities sufficient to give marked protection to trypsin solutions against ionizing radiation.

More troublesome, because more difficult to control, is absorption of volatile chemicals from the atmosphere by the solutions, or any component thereof, before or during irradiation. As was shown in Fig. 4, the addition of as little as one part of ethanol per million (2×10^{-5} M) markedly reduces the amount of inactivation effected by a given dose of radiation. Control experiments clearly demonstrated that, under favorable conditions, solutions can easily absorb from the atmosphere sufficient ethanol to give concentrations of 1 part in 10,000. Other volatile compounds would probably behave in a similar fashion.

It is characteristic of the indirect action of ionizing radiation that the reaction yields are independent of the concentration of solute above a critical concentration level characteristic for each system. Below this level the reaction yields decrease with decreasing concentration, presumably because an appreciable proportion of the active radicals recombine before reacting with the solute molecules. A number of systems are known, however, in which the reaction yields rise continuously with increasing concentrations of solute without reaching a constant level (1, 12–15). The reasons for this are still uncertain; Dale has recently reviewed the various factors that may be involved (16). The inactivation of dilute solutions of trypsin by x-rays falls in the latter category, the reaction yields increasing continuously as the concentration of trypsin is increased. This, in all probability, is not due to an increased contribution to the reaction yields by direct inactivation as the concentration of enzyme is increased, for, if one assumes that the direct action yield for the dissolved state of trypsin is the same as that for the dry state,[10] and that the ionic yield of unity found for the direct inactivation of dry trypsin by deuteron and electron bombardment (9, 21) holds for x-rays, then, even at the highest concentration of trypsin used (1 per cent), the contribution of direct inactivation to the reaction yield would be negligible.

Increased reaction yields with increasing concentrations of trypsin were found regardless of whether 0.005 N hydrochloric, sulfuric, or nitric acid was used as the solvent. The increase was most marked, however, with hydrochloric

[10] This may not be true (16), but it is at present the only means available of estimating the magnitude of the yield to be attributed to direct action.

acid. Perhaps chloride ions compete more effectively for the products of "activated" water, when the concentration of enzyme is low, than do sulfate or nitrate ions. If so, they do it only in the very limited pH range of 2 to 4, and there is no difference in the degree of competition with concentrations of chloride ions between 0.005 and 0.1 M. This is not true with phosphate and acetate buffers, with which progressive inhibition is observed as the concentration of the buffer is increased from 0.001 to 0.1 M. The differences noted between hydrochloric acid and sulfuric or nitric acid were not due to impurities in the hydrochloric acid used, since redistilled hydrochloric acid from a constant boiling mixture (31, 32) gave the same results as non-purified hydrochloric acid. For example, in one of several experiments in which both samples were treated and assayed simultaneously, 0.116 (0.107 to 0.124) micromole of trypsin per liter was inactivated per 1000 r when non-purified hydrochloric acid was the solvent, and 0.117 (0.104 to 0.130) micromole when redistilled acid was used, the original concentrations of trypsin being 2.90 and 2.94 μM respectively.

The mechanisms by which the hydrogen ion concentration of the medium participates in the indirect action of x-radiation are obscure. The pH of maximum efficiency and the over-all results vary with the reaction system being studied (33). Minimum reaction yields for the inactivation of dilute trypsin solutions by ionizing radiations were obtained at approximately pH 7, the isoelectric point of trypsin (34). On the other hand, the direct inactivation of dry trypsin by high intensity electron bombardment was found to be independent of whether the acid (pH 3.4) or the alkaline salt (pH 8.2) was irradiated (21). This suggests that the dependence of the inactivation of trypsin solutions by x-rays on pH may be connected with the state of the trypsin in solution, the reaction yields being low when the zwitter ion structure predominates and high when the cationic or anionic structure prevails.

The temperature dependence of most of the reactions resulting from the indirect action of ionizing radiation is too slight to be noticeable in the limited temperature range in which enzyme solutions can be studied. For example, the reduction of bichromate ions to chromic ions (35) and the oxidation of ferrous ions to ferric ions (36) by x-rays were found to be independent of temperature (to within a few per cent) between 5 and 50°C. A moderate temperature coefficient for the inactivation of trypsin solutions by x-rays was found in this study when the medium in which the trypsin was irradiated was 0.005 N hydrochloric acid. Solutions of trypsin that have been partially inactivated by x-rays continue to lose activity after the radiation treatment is terminated if they are left at 25°C., but do not do so at 2°C. (37). It might be assumed, therefore, that the greater reaction yields obtained at 26°C. were due to the occurrence, during the irradiation process, of this type of radiation-induced secondary inactivation. Several facts refute this interpretation, however. The

maximum period of irradiation used in the temperature studies was 90 minutes. Within this time interval, the reaction yields for the inactivation of dilute solutions of trypsin in 0.005 N hydrochloric acid by x-rays at room temperature were found to be independent of the intensity at which the radiation was delivered (1). Furthermore, the maximum increase in reaction yield that could result from radiation-induced secondary inactivation during 90 minutes, calculated on the basis of the "continued inactivation" previously found in this time interval (37), is 5 per cent, whereas an increase of approximately 50 per cent was found. Moreover, if the differences in the reaction yields found with hydrochloric acid had been due to radiation-induced secondary inactivation, similar differences should have been observed with sulfuric acid, since there is no difference in the rate of "continued inactivation" between trypsin solutions irradiated in 0.005 N hydrochloric acid and those irradiated in 0.005 N sulfuric acid (38). This was not the case. Since no temperature coefficient was found with nitric acid, and since moderate temperature coefficients are generally found in systems in which concurrent competing reactions take place (33), it appears more probable that chloride ions compete for the products of activated water at this pH.

When dilute solutions of trypsin are inactivated by x-rays, exponential dose-reaction curves are usually obtained. They are not found, however, in the presence of some protecting agents; with ethanol, for example, sigmoid curves resembling "multiple-hit" curves (39) are procured.[11] Since the destruction of ethanol by ionizing radiation is a linear function of the dose (24, 41), whereas the inactivation of trypsin is an exponential one, it may be that trypsin and ethanol react simultaneously, thus complicating the kinetics of the inactivation. Minder *et al.* (42) have shown that the reaction-dose curve for the formation of hydrochloric acid from carbon tetrachloride by x-rays is altered by ethanol. In ethanol-free solutions the amount of hydrochloric acid formed is directly proportional to the x-ray dose, but in the presence of from 5 to 50 per cent ethanol it is an exponential function of the dose. The initial reaction yield is not altered appreciably, however. Ethanol also enhances some radiation-induced reactions. Among these are the oxidation of ferrous sulfate (43) and the reduction of ceric sulfate (44), potassium dichromate (35), and diphosphopyridine nucleotide (41). In these cases, however, no change in the reaction-dose relationship was observed, the reaction remaining a linear function of time.

Reaction yields (micromoles enzyme per liter inactivated per 1000 r) of from 0.075 for catalase to 2.9 for alcohol dehydrogenase have been reported (45). It should be emphasized that each of these values was, with few exceptions, obtained only under one set of experimental conditions and applies solely to

[11] Hollaender and his coworkers (40) observed similar changes from exponential to "multiple-hit" curves, when the conditions of growth or irradiation were altered, in their studies on the irradiation of *Escherichia coli* and other organisms.

that set of conditions. Changing the experimental conditions would undoubtedly change the reaction yield in most cases. For example, we have confirmed the finding of Barron *et al.* (22) that the reaction yield for the inactivation of a 0.005 per cent (1.4 μM) solution of trypsin in 0.05 M phosphate buffer, pH 7.5, is 0.05 micromole of trypsin per liter inactivated per 1000 r (ionic yield: 0.018 molecule inactivated per ion pair);[12] but under appropriate experimental conditions reaction yields of 0.9 micromole of trypsin inactivated per liter per 1000 r can be obtained (*cf.* Table I). Any desired intermediate value may be procured by changing the experimental conditions under which the radiation is administered. This illustrates the difficulties encountered in interpreting effects of radiation on enzyme solutions (or other aqueous systems), and in comparing results obtained under different conditions or in different laboratories.

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SUMMARY

The proteolytic activity of dilute solutions of crystalline trypsin is destroyed by x-rays, the amount of inactivation being an exponential function of the radiation dose. The reaction yield increases steadily with increasing concentration of trypsin, varying, as the concentration of enzyme is increased from 1 to 300 μM, from 0.068 to 0.958 micromole of trypsin per liter inactivated per 1000 r with 0.005 N hydrochloric acid as the solvent, from 0.273 to 0.866 with 0.005 N sulfuric acid as the solvent, and from 0.343 to 0.844 with 0.005 N nitric acid as the solvent. When the reaction yields are plotted as a function of the initial concentration of trypsin, they fall on a curve given by the expression $Y \alpha X^K$, in which Y is the reaction yield, X is the concentration of trypsin, and K is a constant equal to 0.46, 0.20, and 0.16, respectively, with 0.005 N hydrochloric, sulfuric, and nitric acids as solvents. The differences between the reaction yields found with chloride and sulfate ions in 1 to 10 μM trypsin solutions are significant only in the pH range from 2 to 4.

The amount of inactivation obtained with a given dose of x-rays depends on the pH of the solution being irradiated and the nature of the solvent. The

[12] Barron *et al.* (22) reported on ionic yield of 0.025 for the inactivation of trypsin under these conditions. This value was apparently calculated for 24 per cent inhibition, ignoring the fact that the inactivation of enzyme solutions by x-rays is usually an exponential function of the dose and not a direct linear one. Recalculation of their data, assuming the inactivation to be exponential in the presence of phosphate buffer, gives an ionic yield of 0.018.

reaction yield–pH curve is a symmetrical one, with minimum yields at about pH 7. Buffers such as acetate, citrate, borate and barbiturate, and other organic molecules such as ethanol and glucose, in concentrations as low as 20 μM, inhibit the inactivation of trypsin by x-radiation. Sigmoid inactivation-dose curves instead of exponential ones are obtained in the presence of ethanol.

The reaction yields for the inactivation of trypsin solutions by x-rays are approximately 1.5 times greater when the irradiation is done at 26°C. than when it is done at 5°C., when 0.005 N hydrochloric acid is the solvent. The dependence on temperature is less when 0.005 N sulfuric acid is used, and is negligible with 0.005 N nitric acid.

The difficulties involved in interpreting radiation effects in aqueous systems, and in comparing the results obtained under different experimental conditions, are discussed.

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