STUDIES ON THE MECHANISM OF ACTION OF IONIZING RADIATIONS

I. Inhibition of Enzymes by X-Rays

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The effect of ionizing radiations on enzyme systems has been the subject of numerous investigations. In the early experiments, however, the amount of radiation necessary to produce inhibition was so high that Scott (1) quite reasonably concluded that x- and gamma rays only influence enzymes when the dose is enormous. The reason for these failures may have been the use of large amounts of enzyme and of impure preparations. In fact, Dale in 1940 (2) by diminishing the concentration of the enzyme, carboxypeptidase, obtained inhibition on irradiation with relatively small doses of x-rays. Dale was the first to postulate that enzyme molecules are not directly affected by the ionizing radiation, but indirectly through collision with a labile product resulting from the ionization of water. This indirect action had already been suggested in 1930 by Risse (3) in his "activated solvent" hypothesis, and was later developed by Fricke (4). Ionizing radiations may act on the protein moiety of the enzyme or on its prosthetic groups. When acting on the protein moiety they may destroy selectively certain groups in the side chain of the molecule that are essential for enzymatic activity, or they may act by breaking hydrogen bonds with production of denaturation or precipitation. When x-rays act on solutes dissolved in water there may result a number of oxidations by the products of ionization of water, such as oxidation of sulfhydryl groups, among the enzymes that require their presence for activity. It is therefore reasonable to assume that these enzymes may be preferentially inhibited on irradiation through oxidation of their sulfhydryl groups to the disulfide. This inhibition would be reversible. If the irradiation dose were increased other groups on the protein might be attacked. In fact, Svedberg and Brohult (5) split hemocyanin into two halves under the action of radiation from radium. The experiments presented in this paper on the reversible inhibition of sulfhydryl enzymes on irradiation with x-rays, and further irreversible inhibition when the dose of x-rays is increased, favor the opinion that sulfhydryl enzymes are the most sensitive to the action of ionizing radiations.

In this, as in subsequent studies on the biological effects of ionizing radiations, we have purposely kept the dose below 5,000 roentgens. In our opinion

the mechanism of action of larger doses of x-rays is more complicated and has no biological interest.

EXPERIMENTAL

Extreme care was taken to have all the water used in these experiments copper-free. Three times distilled water was twice redistilled in all-glass distillation apparatus (pyrex), one containing acid permanganate, the other, barium hydroxide.

Phosphoglyceraldehyde dehydrogenase from rabbit muscle was prepared according to the method of Cori *et al.* (6). Recrystallization of the protein was accomplished by dissolving the centrifuged residue in neutralized 0.02 M glutathione and by addition of 2 volumes of saturated $(NH_4)_2SO_4$. The crystals suspended in $(NH_4)_2SO_4$ containing glutathione and kept at 3° maintained their activity for 1 month. Phosphoglyceraldehyde was prepared according to the method of Meyerhof (7). Some of the phosphoglyceraldehyde used in these experiments was kindly provided by Dr. Meyerhof. Diphosphopyridine nucleotide (DPN) was prepared by the method of Williamson and Green (8). It was 50 per cent pure. The enzyme suspension (1 cc.) was centrifuged at 3° in the high speed centrifuge (15,000 R. P. M.) for 30 minutes. The supernatant fluid was removed with a capillary pipette and the solid was diluted to the appropriate amount in either buffer or in Cu-free water. Na arsenate and Na pyrophosphate were highest purity Kahlbaum samples and were Cu- and Fe-free. Phosphate buffers were made from reagent grade Merck's phosphates. In every case the samples which acted as controls accompanied the irradiated samples to the place of irradiation and were kept at the same temperature. Tests of enzyme activity were performed in a Beckman spectrophotometer in quartz cells, at 25°, by measuring at 3,400 Å the rate of reduction of diphosphopyridine nucleotide in the reaction: phosphoglyceraldehyde + $DPN^+ \rightarrow$ phosphoglyceric acid + DPNH + H^+, which in the presence of arsenate goes to completion. The quartz cells contained 0.1 cc. of enzyme (7 micrograms); 0.3 cc. phosphoglyceraldehyde (4×10^{-7} M); 0.2 cc. DPN 2.5×10^{-7} M); 0.2 cc. Na_2HAsO_4 (6×10^{-6} M); 1 cc. of pyrophosphate buffer pH 8.5 (3.5×10^{-5} M); and water to a total volume of 3 cc. As found by Warburg and Christian (9), for the yeast enzyme when the amount of muscle enzyme was the rate-determining step in the reaction, an exponential curve was obtained on plotting the K[1] values at the end of 1 minute (Fig. 1). These K values were used for the measurement of enzyme activity.

Ribonuclease, five times recrystallized, was kindly provided by Dr. Kunitz. The activity of the enzyme was determined manometrically by measuring the amount of CO_2 produced in bicarbonate buffer, since the enzyme splits ribonucleic acid into smaller acids and thus increases the acidity of the system. Purified commercial nucleic acid (0.01 M) was used as substrate; it was dissolved in bicarbonate buffer pH 7.4, saturated with N_2:CO_2 (95:5). The enzyme was dissolved in water so that 1 cc.

[1] The K values as defined by Warburg and Christian are obtained from the equation $K = \frac{1}{t}\frac{C_0 - C}{C_0 C}$, where t is time in minutes; C_0, the initial concentrtion of DPN, and C, the concentration of DPN at time t.

contained 2.5 micrograms. As can be seen in Fig. 2, the amount of CO_2 liberated was proportional to enzyme concentration from 2.5 to 10 micrograms.

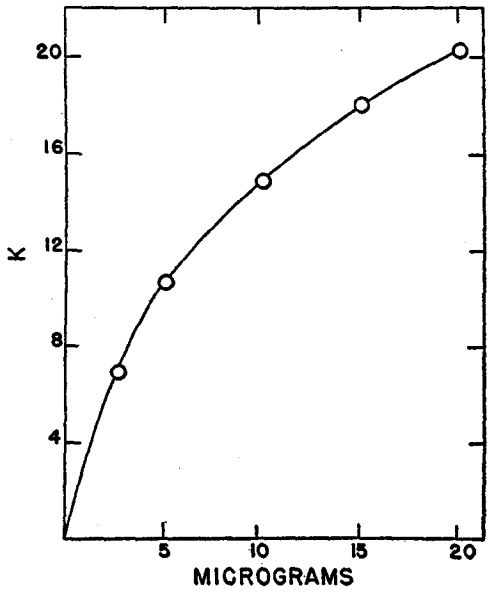

FIG. 1

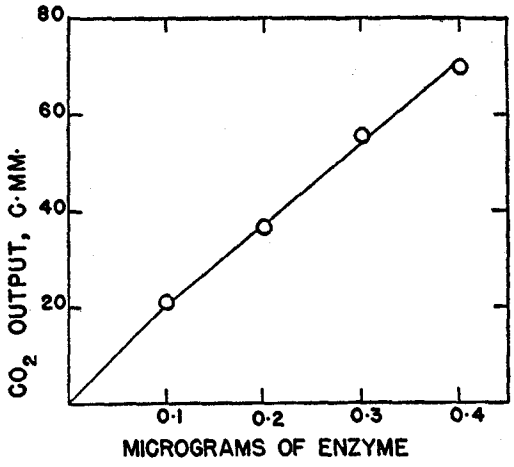

FIG. 2

Crystalline trypsin, three times recrystallized, was kindly provided by Dr. Kunitz. The activity of the enzyme was measured according to Anson (10). The conditions for the test were as follows: urea-hemoglobin substrate, pH 7.5, 2.5 cc.; enzyme in

water, 0.5 cc., or less; water to 3 cc. The samples were incubated for 10 minutes at 25°. Five cc. of 0.3 N CCl_3COOH was added and the samples were filtered 25 minutes later. To 2 cc. of the filtrate were added 4 cc. of 0.5 N NaOH and 1 cc. of the Folin-Ciocalteau reagent. The color produced was read after 5 minutes in the Coleman spectrophotometer at 6,300 Å.

Adenosinetriphosphatase (myosin) was prepared according to the method of Bailey (11). Ten to 40 micrograms per cc. was used in the irradiation experiments. Enzyme activity was determined by measurement of inorganic P after 10 minutes' incubation at 38°.

Succinoxidase from pigeon breast was prepared according to Barron and Singer (12), and enzyme activity was measured by the O_2 uptake on addition of succinate.

Lactic dehydrogenase from beef heart was prepared according to Straub (13). One hundred and sixty-three micrograms of this protein was used in the irradiation tests. Enzyme activity was determined by measurement of O_2 uptake on addition of DPN, flavoprotein, methylene blue, lactate, and cyanide. The purity of the protein moiety was 32 per cent.

Cytochrome oxidase was prepared from pig's heart by the method of Haas (14). Enzyme activity was measured by the O_2 uptake at 38° in the presence of cytochrome C (2.5 mg.); 0.3 cc. of 0.1 M phosphate buffer, pH 7.0; 0.3 cc. of 0.1 M hydroquinone and 1.2 cc. water.

Catalase was prepared according to Dounce (15). Enzyme activity was measured by titration of H_2O_2 with $KMnO_4$ at pH 6.8 and at 0°.

Irradiation was performed at the temperature of crushed ice with a 200 K.V.P. x-ray machine in a General Electric × PT tube (oil-cooled, air insulated). The enzyme solutions were placed in flat bottomed vials (20 mm. high × 16 mm. diameter) and placed in a depression of an aluminum holder which was set on cracked ice. The x-ray dose was measured with Victoreen condenser chambers inserted into the plastic container at the level of the solutions. The vials to be irradiated were placed on a raised plastic floor of a plastic container built with double walls so that cracked ice could be placed around and under the vials. This container was centered under the x-ray tube each time a run was made.

Effect of X-Rays on Sulfhydryl Enzymes

To test the effect of x-rays on the activity of sulfhydryl enzymes, the following sulfhydryl enzymes were chosen: phosphoglyceraldehyde dehydrogenase, which was shown by Rapkine (16) to require the presence of —SH groups for enzyme activity; adenosinetriphosphatase (myosin), which was shown to be a sulfhydryl enzyme by Singer and Barron (17), succinoxidase, demonstrated to be a sulfhydryl enzyme by Hopkins and Morgan (18), and hexokinase.

1. Phosphoglyceraldehyde Dehydrogenase.—For the irradiation experiments 70 micrograms of the crystalline enzyme was used, dissolved in phosphate buffer of different pH values, and of ionic strength, $\mu = 0.2$. The enzyme was irradiated with doses of x-rays varying from 25 r to 500 r. Half-inhibition occurred with 200 r, and almost complete inhibition with 500 r. If this in-

hibition was entirely due to the oxidation of the —SH groups of the enzyme by the products of water irradiation, (the radicals OH and O_2H, and the H_2O_2 and atomic oxygen), the following reversible oxidations could occur:

$$2\ RSH + 2\ OH \rightleftharpoons RS\text{-}SR + 2\ OH^- + 2\ H^+ \quad (1)$$
$$2\ RSH + 2\ O_2H \rightleftharpoons RS\text{-}SR + 2\ H_2O_2 \quad (2)$$
$$2\ RSH + H_2O_2 \rightleftharpoons RS\text{-}SR + 2\ H_2O \quad (3)$$
$$2\ RSH + 2\ O \rightleftharpoons RS\text{-}SR + H_2O_2 \quad (4)$$

It would be possible to reduce the disulfides by addition of glutathione and thus restore enzyme activity. When the enzyme was 21 per cent inhibited, there was complete reactivation on addition of glutathione; when the enzyme

TABLE I

Effect of X-Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase from Rabbit Muscle

Enzyme (70 micrograms in 1 cc.) irradiated in phosphate buffer, pH 7.0 at 0°. Glutathione, 0.002 M. Enzyme activity, $K = \frac{1}{t} \times \frac{C_0 - C}{C_0 C}$ where t, time in minutes; C_0 (DPN) initial; C (DPN) at time t. Temperature 25°.

X-ray dose	Enzyme activity	Inhibition	Reactivation with glutathione
r	$k \times 10^5$	*per cent*	*per cent*
None	4.80		
25	4.80	None	
50	4.80	"	
100	3.81	21	Complete
200	2.43	50	62
300	0.98	80	
400	0.98	80	
500	0.25	94	10

was half-inhibited, there was 62 per cent reactivation; and when the enzyme was completely inhibited, reactivation was only 10 per cent (Table I). In these reactivation experiments enzyme activity was tested immediately after addition of neutralized glutathione (0.002 M). Cori and Cori (19) have pointed out, however, that a time factor is involved in the reaction between the enzyme phosphorylase and glutathione. Whether greater reactivations would have been obtained by allowing more time for complete action is not known.

When the pH of the solution is decreased, the concentration of H_2O_2 formed will increase because a high pH value favors a lower stationary H_2O_2 concentration (20, 21). Furthermore, the oxidizing power of the radicals OH and O_2H, as well as H_2O_2 increases as the H^+ ion concentration increases; hence —SH enzyme inhibition by ionizing radiations will decrease as the pH increases. The experiments in Table II show this to be the case.

If the inhibiting effect of x-rays on this enzyme is mainly due to oxidation

of —SH groups, it will be possible to protect the enzyme by previous addition of glutathione. The experiments in Table III show that previous addition of glutathione reduced the inhibition from 50 per cent to 9 per cent. Addition of diphosphopyridine nucleotide had no effect at all.

2. Yeast Hexokinase.—The enzyme hexokinase which catalyzes the reaction glucose + ATP $\rightleftharpoons$ glucose-6-phosphate + ADP has been prepared in crystalline form from yeast by Kunitz (22) and by Berger *et al.* (23). The crystalline yeast hexokinase was kindly provided by Dr. Kunitz. Hexokinase is an —SH

TABLE II

Effect of pH on the Inhibition of Phosphoglyceraldehyde Dehydrogenase by X-Rays

Buffer, phosphate; x-ray dose, 400 r.

pH	Activity		Inhibition
	Control	X-ray	
	$k \times 10^5$	$k \times 10^5$	*per cent*
6.2	12.2	3.86	63
8.3	16.6	10.4	32

TABLE III

Inhibition of Phosphoglyceraldehyde Dehydrogenase by X-Rays Protection with Glutathione

X-ray dose, 200 r; pH, 7.0.

Experimental conditions	Activity	Inhibition
	$k \times 10^5$	*per cent*
Control	4.8	—
Enzyme treated with x-rays	2.43	50
" " + glutathione, 1.8×10^{-3}M	4.33	9
" " + DPN, 2.4×10^{-4}M	2.43	50

enzyme, as demonstrated by inhibition with sulfhydryl reagents. The enzyme was treated with an —SH oxidizing agent, iodosobenzoate; an alkylating agent, iodoacetamide; and a mercaptide-forming agent, *p*-chloromercuribenzoate. Iodosobenzoic acid inhibited 15 per cent; iodoacetamide, 23 per cent; and *p*-chloromercuribenzoate, 89 per cent. In agreement with the low inhibiting effect of the oxidizing agent, iodosobenzoic acid, irradiation by x-rays had little effect on the enzyme activity. Irradiation with 1,000 r inhibited 13 per cent, while 2,000 r inhibited 18 per cent (Table IV).

3. Adenosinetriphosphatase (Myosin).—While the enzyme activity of myosin is easily and accurately determined, under the conditions of these experiments extreme precautions had to be taken to secure reliable data. Rapid inactiva-

tion of the enzyme, when in dilute solutions and at high pH values, and the variation found in duplicate and triplicate experiments on the extent of inhibition produced by a given dose of x-rays had to be considered. For these reasons, when partial inhibition occurred, at least six samples were measured.

TABLE IV

Effect of Sulfhydryl Reagents and of X-Rays on the Activity of Yeast Crystalline Hexokinase

Enzyme activity measured at pH 7.9, veronal-acetate buffer. 12 micrograms of enzyme; 0.1 M glucose; 0.1 M adenosinetriphosphate. Temperature 20°. Time of incubation, 5 minutes. E (log $\frac{I_0}{I}$) proportional to 7 minutes hydrolyzable P which has been transferred to glucose in the reaction: glucose + ATP = glucose-6-phosphate + ADP.

System	E	Inhibition
		per cent
Enzyme—control	0.112	
" + *p*-ClHg benzoate, 1×10^{-4} M	0.012	89.5
" + iodoacetamide, 1×10^{-3} M	0.086	23.5
" + iodosobenzoate, 1×10^{-3} M	0.095	15
" + x-rays, 1000 r	0.098	12.5
" + " 2000 r	0.091	18.7

TABLE V

Effect of X-Rays on the Activity of Adenosinetriphosphatase (Myosin)

Nitrogen content of preparation used for irradiation, 0.02 mg. per cc. Buffer, bicarbonate-carbonate, 0.01 M, pH 9.1. The P figures give the amount formed in 10 minutes' incubation at 38°.

X-ray dose	Phosphorus		Inhibition
	Control	X-ray	
r	*micrograms*	*micrograms*	*per cent*
10	21.4	18.9	10
50	21.4	17.0	20
100	21.4	14.6	32
250	21.4	8.6	60
500	21.4	8.0	63
1000	21.4	1.1	95

When myosin was irradiated at pH 9.1 (bicarbonate-carbonate buffer), an inhibition was noticed after irradiation with 10 r and was complete with 1,000 r (Table V).

When myosin preparations are kept at 3° for a week (aged myosin) the enzyme activity is more sensitive to the inhibiting effect of mild oxidizing agents. The same increased sensitivity was observed towards x-rays. Aged myosin became so sensitive that 1 to 10 r produced marked inhibitions. Thus, 10

r and 8 r produced in excess of 50 per cent inhibition, while 1 r inhibited 30 per cent (Table VI).

Addition of glutathione produced a reactivation of the enzyme. Enzyme inhibition with 100 r was almost completely reversed on addition of glutathione. When the x-ray dose was increased to 500 r, reactivation by glutathione reached only 50 per cent (Table VII).

TABLE VI

Inhibition of Adenosinetriphosphatase (Aged Myosin) by Low Doses of X-Rays

Buffer, bicarbonate-carbonate, pH 9.1. Nitrogen content, 0.02 mg. per cc. Phosphorus liberated in 10 minutes at 38°.

X-ray dose	Phosphorus		Inhibition
	Control	X-ray	
r	*micrograms*	*micrograms*	*per cent*
1	11.9	8.4	30
5	11.9	5.4	55
10	11.9	5.7	52

TABLE VII

Adenosinetriphosphatase Inhibition by X-Rays
Reactivation with Glutathione

Buffer, bicarbonate-carbonate, pH 9.1. Nitrogen content of myosin, 0.02 mg. per cc. Glutathione (GSH), 0.02 M.

X-ray dose	Inhibition	Reactivation
	With x-ray	X-ray + GSH
r	*per cent*	*per cent*
10	10	80
100	22	64
500	41	56
1000	73	22

4. Succinoxidase.—Havard (24) reported that x-rays had slight effect on the activity of succinoxidase when irradiated with a dose of 20,000 r. These negative results were probably obtained because in the tissue suspensions the enzyme was not the rate-limiting step in the reaction. For the experiments on irradiation of succinoxidase, it was established first that 0.5 cc. of the enzyme suspension in phosphate buffer, pH 7.0, gave maximum O_2 uptake in the presence of 0.05 M succinate. With smaller amounts of enzyme the O_2 uptake was directly proportional to the enzyme concentration. Amounts of enzyme ranging from 0.4 cc. to 0.05 cc. were diluted to 1 cc. with phosphate buffer and were irradiated with 5,000 r. Inhibition was small when 0.2 to 0.4 cc. of the

enzyme suspension was taken. With 0.1 cc. of enzyme the inhibition rose to 29 per cent; with 0.05 cc. inhibition was complete (Table VIII). In the presence of glutathione, the inhibition dropped to 23 per cent. With glutamate (used by Dale (25) to protect *d*-amino acid oxidase from inhibition by x-rays), the inhibition was 62 per cent (Table VIII). X-ray inhibition was reversible.

TABLE VIII

Effect of X-Rays on the Activity of Succinoxidase

Buffer, phosphate, 0.02 M, pH 7.0; succinate, 0.05 M. Temperature 38°. Dose of x-rays, 5000 r. O_2 uptake in 50 minutes. Glutathione, 0.005 M.

Amount of enzyme	O_2 uptake		Inhibition
	Control	X-rays	
cc.	*c.mm.*	*c.mm.*	*per cent*
0.4	290	270	7
0.3	235	214	9
0.2	147	132	10
0.1	82	58	29
0.05	25	0	Complete
0.05 + glutathione	34	26	23
0.05 + glutamate	26	10	62

TABLE IX

Inhibition of Succinoxidase by X-Rays
Reactivation with Glutathione

X-ray dose, 5000 r. Glutathione, 0.005 M added after irradiation.

Enzyme	Inhibition	Reactivation
	With x-ray	X-ray + GSH
cc.	*per cent*	*per cent*
0.07	75	94
0.06	77	75
0.05	Complete	42

Addition of glutathione after irradiation reactivated the enzyme to varying degrees (Table IX).

Effect of X-Rays on Non-Sulfhydryl Enzymes

For the study of the effect of x-rays on enzymes which do not require the presence of —SH groups for enzyme activity the following were used: trypsin, ribonuclease, and catalase, all of them in crystalline form, and lactic dehydrogenase and cytochrome oxidase.

1. Trypsin.—X-ray irradiation of impure trypsin (Fairchild's powdered

trypsin) was performed by Hussey and Thompson (26), who reported no effect from irradiation by large amounts of x-ray (2 hours in a 140 kv. machine, 4 ma. at a distance of 30 cm.).

Fifty micrograms of the enzyme was irradiated at two pH values, 7.5 and 9.1. There was no effect at all with 1,000 r, while 5,000 r inhibited 25 per cent (Table X).

TABLE X

Effect of X-Rays on the Activity of Crystalline Trypsin

Buffers, phosphate 0.05 M, pH 7.5 and bicarbonate-carbonate, 0.01 M, pH 9.1. Enzyme 50 micrograms per cc. E values ($\log \frac{I_0}{I}$) proportional to protein hydrolysis and enzyme activity.

X-ray dose	pH	E		Inhibition
		Control	X-ray	
r				*per cent*
1000	7.5	0.335	0.335	None
5000	7.5	0.335	0.255	24
1000	9.1	0.332	0.345	None
5000	9.1	0.332	0.245	26

TABLE XI

Effect of X-Rays on Crystalline Ribonuclease

Buffer, bicarbonate-carbonate, pH 9.1. Gas phase N_2:CO_2. Enzyme activity measured by CO_2 evolution on hydrolysis of ribonucleic acid.

Enzyme	X-ray dose	CO_2 output		Inhibition
		Control	X-rays	
micrograms	*r*	*c.mm.*	*c.mm.*	*per cent*
25	1000	270	257	7
20	5000	256	189	26
10	5000	186	119	36
7.5	5000	163	92	44
5.0	5000	128	51	60

2. Ribonuclease.—This crystalline enzyme was irradiated at different concentrations. When 25 micrograms in 1 cc. was irradiated with 1,000 r and with 5,000 r there was no effect. When the amount of enzyme was progressively decreased while the amount of x-ray irradiation was kept constant, the inhibition increased steadily, from 26 to 60 per cent (Table XI). Undoubtedly this inhibition was due to protein denaturation, which usually occurs with high dose irradiation. Lea *et al.* (27) irradiated *dried* ribonuclease and found that irradiation of 2 mg. with 3.4×10^7 r destroyed 37 per cent of the activity of the enzyme.

3. Lactic Dehydrogenase.—The enzyme (163 micrograms in 1 cc.) was irradiated at two pH values, 7.2 and 9.1, and in two doses, 1,000 r and 5,000 r. An aliquot (0.07 cc.) was taken for measurement of activity. Enzyme inhibition was negligible (Table XII).

4. Cytochrome Oxidase.—Preliminary experiments showed that 0.05 cc. to 0.2 cc. of the cytochrome oxidase gave a linear relationship between O_2 uptake and enzyme concentration. For irradiation the original enzyme solution was

TABLE XII

Effect of X-Rays on Lactic Dehydrogenase

Buffers, 0.05 M phosphate, pH 7.5 and bicarbonate-carbonate, 0.01 M, pH 9.0. Enzyme, 163 micrograms per cc. for irradiations.

X-ray dose	pH	O_2 uptake		Inhibition
		Control	X-ray	
r		*c.mm.*	*c.mm.*	*per cent*
1000	7.2	56.8	53.5	5.6
5000	7.2	56.8	53.0	6.5
1000	9.0	31.7	31.9	None
5000	9.0	31.7	30.3	4

TABLE XIII

Effedt of X-Rays on Cytochrome Oxidase

Dose, 5000 r. Original enzyme diluted for irradiation.

Dilution of enzyme	O_2 uptake in 10 min.	
	Control	X-rays
	c.mm	*c.mm.*
1:5	173.1	166.3
1:10	103.2	102
1:15	45.0	43.0
1:20	25.5	25.7

diluted so as to contain the equivalents of 0.05, 0.1, 0.15, and 0.2 cc. of the original sample. The diluted samples were irradiated with 5,000 r at pH 7.0. No significant inhibition was obtained on irradiation with 5,000 r even at the lowest dilution used (Table XIII).

5. Catalase.—Tytell and Kersten (28) have reported that catalase (a non-sulfhydryl enzyme) is quite resistant to the action of x-rays, while urease (a sulfhydryl enzyme) was more easily inhibited. Four samples of crystalline catalase containing 9.0, 11.3, 15.0, and 22.5 micrograms of enzyme per cc. were irradiated with 5,000 r. 0.5 cc. of these irradiated samples was taken for the determination of enzyme activity. No inhibition was detected even at the highest dilution (Table XIV).

6. Ionic Yields.—It is customary in radiation chemistry to express the efficiency of a radiation in initiating chemical changes as the ratio of the number of molecules (M) which are decomposed to the number of ion pairs (N) produced in the system. The ratio $\frac{M}{N}$ is the ionic yield of the reaction. In gas reactions the most striking feature is the large number of molecules which can sometimes be brought into reaction compared with the number of molecules ionized.

TABLE XIV

Effect of X-Rays on Catalase

Dose, 5000 r. H_2O_2, 0.005 N. Figures give cubic centimeters of H_2O_2 destroyed by 5 cc. of enzyme solution in 5 minutes.

Enzyme	H_2O	
	Control	X-rays
micrograms	*cc.*	*cc.*
9.0	1.72	1.97
11.3	1.20	1.14
15.0	1.08	0.98
22.5	0.69	0.70

TABLE XV

Ionic Yields of Enzymes Inhibited by X-Rays

Enzyme	Molecular weight	Ionic yield
Phosphoglyceraldehyde dehydrogenase	70,000	0.93
Trypsin	36,500	0.025
Ribonuclease	13,000	0.03
Carboxypeptidase*	35,000	0.16
d-Amino acid oxidase‡	70,000	0.1

* From the manuscript of Dale, Meredith, and Gray quoted previously.

‡ From Lea's calculation (29).

These reactions with high ionic yields belong in most cases to the class of chain reactions. The ionic yield for carboxypeptidase inhibited by x-ray irradiation, as calculated by Dale, Meredith, and Gray,[2] is 0.16; that of *d*-amino acid oxidase, 0.1.

For the calculation of the ionic yields in Table XV the values of 1.616×10^{12} ion pairs liberated in 1 cc. of water per 1 r, and of 6.023×10^{23} for Avogadro's number, have been taken. There are, as yet, no reliable data for the molecular weight of phosphoglyceraldehyde dehydrogenase; it has been assumed to be 70,000. Since 70 micrograms per cc. of solution was irradiated, there would be

[2] Dale, W. M., Meredith, W. J., and Gray, L. H., The inactivation of an enzyme (carboxypeptidase) by x- and α radiation. Manuscript kindly sent to one of us by Dr. Gray.

6.023×10^{14} molecules of enzyme. Irradiation with 200 r (3.23×10^{14} ion pairs) produced half-inhibition, *i.e.* "destruction" of 3.01×10^{14} molecules, which gives an ionic yield of 0.93. Unfortunately, recent studies favor the opinion that adenosinetriphosphatase is a small protein adsorbed in myosin; hence, no ionic yields can be calculated from our data. The ionic yields of two non-sulfhydryl enzymes, trypsin and ribonuclease, are 0.025 and 0.03 respectively. The ionic yield for ribonuclease is in remarkable agreement with that given by Lea (29) from unpublished data of Lea and Holmes (ionic yield, 0.03).

DISCUSSION

The experiments presented here have shown that when dilute aqueous solutions of enzymes are irradiated with x-rays, the sulfhydryl enzymes, *i.e.* those requiring —SH groups in the protein moiety for enzymatic activity, are more susceptible to inhibition than enzymes which need no —SH groups for activity. It is postulated that inhibition of sulfhydryl enzymes was produced by oxidation of the sulfhydryl groups. The mechanism of this oxidation can best be explained by following Weiss's suggestion (30) of the series of reactions which might occur when water is irradiated with x-rays. The products first formed are the positive ion H_2O^+ and an electron:

$$H_2O \xrightarrow{\text{radiation}} H_2O^+ + e \tag{5}$$

Because of the high energy of hydration of H^+, the reaction

$$H_2O^+ \rightarrow H^+ + OH \tag{6}$$

is highly exothermic and probably occurs soon after ionization. There is no likelihood of its persisting long enough to combine with the electron to form water again. The electron which was set free at the ionzation will react with water:

$$H_2O + e \rightarrow H_2O^- \tag{7}$$

Decomposition of the H_2O^- ion by the exothermic reaction will give:

$$H_2O^- \rightarrow H + OH^- \tag{8}$$

Furthermore, the following reactions are possible:

$$OH + OH \rightarrow H_2O_2 \tag{9}$$
$$H + H \rightarrow H_2 \tag{10}$$
$$OH + OH \rightarrow H_2O + 0 \tag{11}$$
$$2\,0 \rightarrow O_2 \tag{12}$$
$$H_2 + OH \rightarrow H_2O + H \tag{13}$$
$$H_2O_2 + H \rightarrow H_2O + OH \tag{14}$$
$$H + O_2 \rightarrow HO_2 \tag{15}$$
$$H_2O_2 + OH \rightarrow H_2O + HO_2 \tag{16}$$
$$HO_2 + HO_2 \rightarrow H_2O_2 + O_2 \tag{17}$$
$$HO_2 + H \rightarrow H_2O_2 \tag{18}$$
$$H_2O_2 + HO_2 \rightarrow H_2O + O_2 + OH \tag{19}$$

When oxygen is present in water there may occur a reduction of the molecule which, if it happens by univalent steps, according to Michaelis (31) there would be formation of

$$O_2 \rightarrow O_2^- \rightarrow O_2^- \rightarrow O_2^= \rightarrow O_2^= \tag{20}$$

In water, where protons can be added, these would be converted to

$$O_2 \rightarrow O_2H \rightarrow O_2H_2 \rightarrow OH + OH_2 \rightarrow 2\ OH_2 \tag{21}$$

Reduction of molecular oxygen would thus produce three powerful oxidizing agents, the radicals OH and O_2H, and H_2O_2.

There are four powerful oxidizing agents among these series of reactions: the free radicals OH and O_2H, atomic oxygen, and H_2O_2. Oxidation of the sulfhydryl groups of the protein by these agents will take place as indicated in Equations 1 to 4. Furthermore, the H_2O_2 produced during this oxidation will itself act as an oxidizing agent, thus increasing the yield of the reaction. Evidence for the occurrence of these oxidations was provided by enzyme reactivation on addition of glutathione, glutathione converting the disulfide groups back into the sulfhydryl stage. However, complete reactivation was possible only when small amounts of radiation were used and when inhibition was partial. As the dose of x-rays increased and inhibition became more complete, the degree of reactivation diminished. For example, irradiation of phosphoglyceraldehyde dehydrogenase with 500 r produced an inhibition of 50 per cent, and reactivation of 62 per cent on addition of glutathione. With this amount of irradiation, therefore, 62 per cent of the total inhibition was produced by oxidation of the sulfhydryl groups (reversible inhibition) and 38 per cent must have been produced by denaturation of the protein through rupture of the hydrogen bonds or oxidation of other oxidizable groups of the protein (irreversible inhibition). These two processes are different and are evidence that ionizing radiations in small amounts act specifically on the sulfhydryl enzymes by oxidation of the —SH groups *without destruction* of the protein molecule, while large amounts produce non-specific, protein denaturation.

Proteins possess two kinds of —SH groups: (1) freely reacting —SH groups, easily oxidized by mild oxidizing agents; and (2) *sluggish* —SH groups which are not oxidized by these agents, but react with mercaptide-forming agents (Hg, As, Cd, etc.). The sulfhydryl groups of hexokinase seem to contain these two kinds of —SH groups. The freely reacting —SH groups of hexokinase seem to be only about 15 per cent of the total —SH groups, as calculated from the inhibition with iodosobenzoate (mild oxidizing agent). Inhibition of the enzyme with x-rays (1,000 to 2,000 r) was only 12 to 18 per cent. In spite of the high oxidizing power of the OH and O_2H radicals, x-rays in moderate doses did not oxidize those sluggish —SH groups which could not be oxidized by iodosobenzoate.

The effect of very low doses of x-rays on aged preparations of adenosine-triphosphatase is quite remarkable since inhibition of enzyme activity was observed even after irradiation with 1 r. Such inhibitions are further proof that ionization of water by "one ion pair" starts a chain reaction and that several of the radicals produced react with the sulfhydryl groups of the enzyme. They also speak against the postulated short life of the radicals formed on ionization of water.

Inhibition of non-sulfhydryl enzymes by x-rays required a greater amount of x-ray irradiation per molecule of enzyme. Whether these inhibitions are due simply to the denaturation of the protein through rupture of hydrogen bonds by the products of the irradiation of water, or are due to chemical action on certain side chains of the molecule necessary for enzyme activity, cannot be answered.

SUMMARY

Dilute solutions of sulfhydryl enzymes (phosphoglyceraldehyde dehydrogenase, adenosinetriphosphatase, succinoxidase) showed reduced activity on irradiation by small amounts of x-rays. When the inhibition was partial the enzyme was reactivated on addition of glutathione. When the inhibition was more complete, reactivation was only partial. These observations are interpreted as being due to oxidation of the —SH groups of the protein by the products of water irradiation, the radicals OH and O_2H, and H_2O_2 and atomic oxygen. The irreversible inhibition which occurs when the dose of x-rays is increased is attributed to protein denaturation.

Inhibition of the non-sulfhydryl enzymes trypsin, catalase, and ribonuclease, which required larger amounts of x-rays, is attributed to protein denaturation.

These experiments are further evidence that inhibition of enzymes by ionizing radiations is due to the indirect action of the products of irradiated water rather than to direct ionization of the enzyme through collision with the ionizing radiation.

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