STUDIES ON THE MECHANISM OF ACTION OF IONIZING RADIATIONS

II. Inhibition of Sulfhydryl Enzymes by Alpha, Beta, and Gamma Rays

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In the preceding paper (1) it was shown that dilute aqueous solutions of sulfhydryl enzymes are inhibited by small doses of x-rays by oxidation of the —SH groups of the protein moiety. Hardly any studies have been made on the effect of other ionizing radiations. Northrop (2), who studied the inactivation of crystalline pepsin by beta and gamma rays from radium, reported that inactivation required large amounts of radiation. Presented in this paper are experiments on the effect of alpha, beta, and gamma radiations on the activity of two crystalline sulfhydryl enzymes, phosphoglyceraldehyde dehydrogenase and urease. Enzyme inhibition by these radiations was produced by the same mechanism as that of x-rays; *i.e.*, oxidation of the —SH groups by the products of water irradiation.

EXPERIMENTAL

The water was purified with the same precautions as those indicated in the preceding paper (1).

The source of alpha radiation was a solution of citrate buffer containing 30 microcuries per cc. of polonium. One cc. was diluted to 10 cc. with 0.2 M phosphate buffer, pH 7.0. The irradiated tubes received 0.4 cc. of this solution to a total of 2.1 cc. The final polonium concentration was 0.57 microcuries per cc. This amount of radiation was calculated to give 180 r per day per cc.

The source of beta radiation was a solution of $Sr^{89}Cl_2$ containing 250 microcuries per cc. One cc. was diluted to 11.25 cc. with citrate buffer, pH 7.0, and this in turn was diluted ten times more with 0.2 M phosphate buffer, pH 7.0. For irradiation, 0.4 cc. of this solution was added to a total volume of 2.1 cc. The final concentration of Sr^{89} was 0.42 microcuries per cc. This amount of radiation was calculated to give 14.6 r per day per cc.

The source of gamma rays was a 1 gm. sample of radium enclosed in a brass tube with a long piece of silk fishing line attached to one end. This was kept in a lead brick cave. The line led through a hollow aluminum tube to a pulley fastened to the ceiling (Fig. 1). Before the experiments started, the source was raised to the ceiling and the aluminum tube shifted from the cave to a lusteroid test tube placed in the center of the holder containing the test tubes to be irradiated. The source

was then lowered into position, and the time of irradiation was measured with a stop watch. The sample holder was made up of two round half-inch pieces of lucite with a hole in the middle of each to hold the tube in which the source was stationed. A series of holes at 5 cm. radius from the center held the pyrex tubes containing the enzyme. One hole of this set was enlarged to accommodate a test tube that held the Victoreen dosimeter (Fig. 2). A series of tests with a 250 r capacity dosimeter indicated a rate of gamma ray emission of 4.5 r per minute at 5 cm. distance. The holder fitted smoothly into a half-gallon Dewar flask that contained sufficient cracked

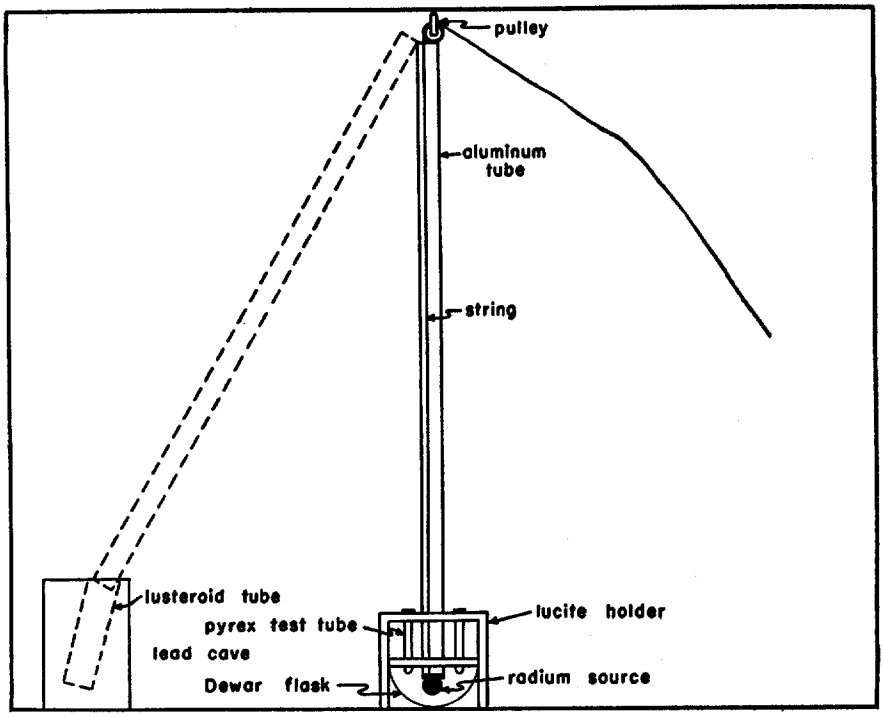

FIG. 1. Apparatus for the irradiation of enzymes with gamma rays.

ice and water to surround the solutions in the test tubes. A piece of wood in the bottom of the central tube maintained the level of the source at the same height at the bottom of the tubes containing the enzyme.

Urease was prepared from arlco jack bean meal by a modification of the method of Hellerman *et al.* (3). The first crop of crystals was dissolved in 1×10^{-2} M neutralized glutathione instead of water. Urease activity was determined by a modification of the method of Van Slyke and Archibald (4). It was found that addition of 0.1 cc. of 1 M glycine to the urea-phosphate solution acted as an effective substitute for the egg albumin recommended by these authors. Quantitative recoveries of added urease were easily secured when glycine was added to the substrate. The

determination of urease activity was carried out at the temperature of the laboratory (24–28°); the correction factor of Van Slyke and Archibald was utilized in the calculation of urease units. The incubation period was 15 minutes, followed by addition of saturated carbonate and aeration for 30 minutes into 4 per cent boric acid. The liberated ammonia was titrated with 0.02 N HCl, using the mixed indicator of Sobel *et al.* (5).

The Sulfhydryl Groups in Phosphoglyceraldehyde Dehydrogenase.—The presence of —SH groups on this enzyme was concluded from Rapkine's experiments in muscle suspensions (6), but it was necessary to demonstrate their presence

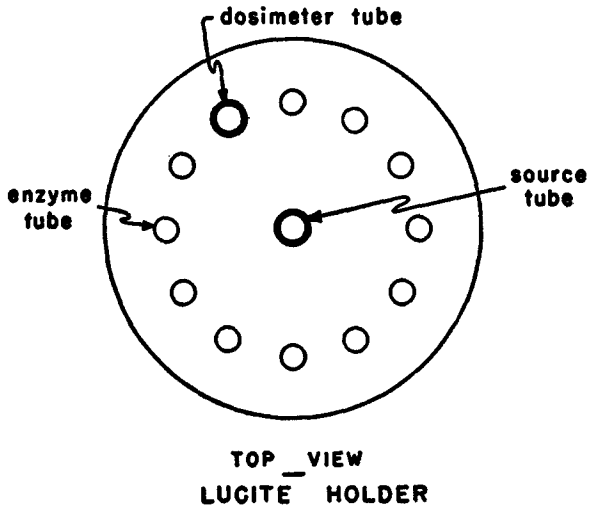

FIG. 2. Lucite holder where the test tubes containing enzyme solutions are kept when irradiated with gamma rays.

in the crystalline enzyme. A solution of the enzyme (17 micrograms) in phosphate buffer, pH 7 ($\mu = 0.2$) was treated with increasing amounts of *p*-chloromercuribenzoate (0.001 M). At the end of 30 minutes, the enzyme activity of an aliquot was measured. Enzyme inactivation was proportional to *p*-chloromercuribenzoate addition (Table I). From extrapolation of these activity titrations, it was calculated that 1 gm. of enzyme contained 0.79 mM —SH groups. Reactivation of the enzyme was attempted by addition of glutathione immediately before the determination of enzyme activity. Under these conditions glutathione failed to produce complete reactivation of the enzyme. This is the first case where inhibition by a mercaptide-forming agent was not reversed on addition of glutathione. The —SH groups of native and duponol PC-denatured enzyme were also determined with Anson's ferricyanide method (7) (Table II). From these titrations it can be concluded that

the native protein contains 0.23 mM and the denatured protein, 0.82 mM of —SH groups per gm. From a comparison of the titration of enzyme activity with *p*-chloromercuribenzoate with the titration of the total —SH groups in the denatured protein, it may be concluded that most of the —SH groups of the protein (93 per cent) are necessary for enzyme activity.

Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase.— Polonium was chosen as the source for alpha ray emission because it emits alpha rays of energy 5.298 e.m.v.[1] with practically no other radiation. The test tubes

TABLE I

Inhibition of Phosphoglyceraldehyde Dehydrogenase by p-chloromercuribenzoate Effect of Glutathione

p-Cl-Hg benzoate	*K* values		Inhibition
	Without GSH	With GSH	
cc. of 1 × 10⁻³ M	× 10⁵	× 10⁵	*per cent*
0	7.5	7.5	
0.05	4.8	4.8	36
0.10	1.6		79
0.12	0.7		90
0.20	0	0	Complete

TABLE II

The Sulfhydryl Groups of Native and Denatured Phosphoglyceraldehyde Dehydrogenase

Sulfhydryl groups measured by ferricyanide titration. The figures give micromoles —SH per gm. protein.

Sample	SH groups in protein	
	Native	Denatured
	micromoles	*micromoles*
I	0.246	0.817
II	0.22	0.80
III	0.22	0.81

containing enzyme and polonium were kept with the control test tubes at the temperature of cracked ice in a room at 3°. The enzyme activity of the control solutions remained unimpaired for 6 days, while the activity of the solutions containing polonium decreased steadily, so that at the end of 6 days enzyme activity had entirely disappeared (Table III). These experiments demonstrate that alpha rays are as effective as x-rays in inhibiting this sulfhydryl enzyme. Evidence that this inhibition is partly due to oxidation of the —SH groups of the protein was obtained by the measurement of enzyme activity after the addition of glutathione (0.01 M neutralized glutathione added simultaneously

[1] e.m.v. signifies 10^6 e.v. (electron volts).

to the control and to the irradiated samples); a partial reactivation—from 26 to 32 per cent—was always obtained (Table IV).

When alpha rays irradiate water, a definite amount of H_2O_2 is produced, which according to Frilley (8) is 0.54 molecules per ion pair in the solution. In order to separate the contribution of H_2O_2 from that of the radicals OH and O_2H in the enzyme inhibition, 1 microgram of catalase was added to the test

TABLE III

Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase

Enzyme activity determined by the value of $K = \frac{1}{t} \times \frac{C_0 - C}{C_0 C}$ where t is time in minutes; C_0, initial concentration of DPN (2.5×10^{-7} M); C, concentration of DPN at time t.

Irradiation		K values		Inhibition
		Control	Polonium	
days	*r*	× 10^5	× 10^5	*per cent*
1	180	10	4.15	58
2	360	10	2.7	73
4	720	10	0.8	92
6	1080	10	0	Complete

TABLE IV

Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase Reactivation with Glutathione

The control K values were those obtained after addition of glutathione.

Irradiation 0.57 microcuries per cc.	Inhibition by α rays	K values		Inhibition
		After glutathione		
		Control	Polonium	
days	*per cent*	× 10^5	× 10^5	*per cent*
1	58	14.6	8.3	43
2	73	13.5	6.8	50
4	92	13.5	4.7	65
6	Complete	11.8	3.8	68

tubes previous to the addition of polonium. Catalase protected the enzyme partially from the inhibitory action of alpha rays, the protection being from 41 to 56 per cent of the total inhibition (Table V). This protective action of catalase is due to the destruction of H_2O_2 formed on irradiation and not to the protection reported by Dale (9), because crystalline egg albumin added at a molar concentration 1,000 times greater than that of catalase (18 micrograms) had no effect at all.

Effect of Beta Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase.—Beta rays acted as powerful inhibitors of the enzyme, for inhibition was

observed even after irradiation with 14 r (1 day) (Table VI). However, reactivation of the enzyme was not obtained on addition of glutathione.

TABLE V

Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase Protection with Catalase

Irradiation 0.57 microcuries per cc.	Inhibition by α rays	K values		Inhibition
		Catalase addition		
		Control	Polonium	
days	*per cent*	$\times 10^5$	$\times 10^5$	*per cent*
1	58	10.7	7.6	24
2	73	11.5	6.8	32
4	92	10.4	4.7	53
6	Complete	10	3.5	65

TABLE VI

Effect of Beta Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase

Irradiation 0.42 microcuries per cc.		K values		Inhibition
		Control	β rays	
days	*r*	$\times 10^5$	$\times 10^5$	*per cent*
1	14.6	10	9	10
2	29	10	8.3	17
4	56.5	10	6.8	32
6	88	10	6.3	37

TABLE VII

Effect of Beta Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase Protection with Catalase

Phosphoglyceraldehyde dehydrogenase, 140 micrograms; catalase, 1 microgram. $Sr^{89}Cl_2$ 0.888 microcuries. Volume, 2.1 cc.

Irradiation	Inhibition	K with catalase		Inhibition
		Control	β ray	
days	*per cent*	$\times 10^5$	$\times 10^5$	*per cent*
1	10	10.7	10	6.5
2	17	11.5		
4	32	10.4	10	4
6	37	10	9.5	5

Previous addition of catalase protected the enzyme effectively, especially after prolonged irradiation (Table VII). No explanation can be offered for the lack of enzyme reactivation.

Effect of Gamma Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase.—In these experiments a very dilute solution of enzyme was used, five times less than in the previous experiments. Half-inhibition was produced on irradiation with 50 r (Table VIII). Addition of glutathione after irradiation produced no reactivation.

Effect of Gamma Rays on the Activity of Urease.—For the irradiation of urease with gamma rays the experiments were performed at first in the presence of glycine (0.1 M), because addition of glycine allowed more quantitative deter-

TABLE VIII

Effect of Gamma Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase

Amount of enzyme 14 micrograms. Buffer, phosphate 0.02 M; pH, 7.

Dose	*K* value	Inhibition
r	× 10^5	*per cent*
None	4.2	
25	3.5	17
50	2.0	54
200	1.4	67

TABLE IX

Inhibition of Urease by Gamma Ray Irradiation
Protection with Glycine

Urease, 1.7 micrograms in 1.1 cc. phosphate buffer, pH 7. Enzyme activity given in units. Unit as defined by Sumner and Hand (18).

Dose	Glycine	Control	Gamma rays	Inhibition
r	M	*units*	*units*	*per cent*
100		1,000	760	24
200		1,000	705	30
100	10^{-1}	1,190	1,196	None
100	10^{-2}	1,190	1,159	"
100	10^{-4}	1,190	1,137	4.5
100	10^{-6}	1,190	1,079	9

minations of enzyme activity. It was found, however, that glycine protected the enzyme from inhibition. A concentration of 10^{-2} M protected it completely, while in the absence of glycine 100 r produced an inhibition of 24 per cent. Even 10^{-6} M glycine protected 9 per cent (Table IX).

The mechanism of this protective action is not known. It must be recalled that Bailey (10) found that the enzyme activity of adenosinetriphosphatase (another sulfhydryl enzyme) was considerably enhanced on addition of glycine and other amino acids. Glycine is known to form complex salts with heavy metals.

Enzyme inhibition by gamma rays was not released on addition of glutathione. This lack of reactivation was taken advantage of to demonstrate definitely that inhibition of the enzyme by gamma rays is due to oxidation of the sulfhydryl groups of the protein. Hellerman *et al.* (3) showed that *p*-chloromercuribenzoate inhibits urease by combination with the —SH groups (formation of the compound R-S-Hg-benzoate), and that inhibition is released on addition of a sulfhydryl-containing substance. If enzyme inhibition by gamma rays were due only to oxidation of the —SH groups by the oxidizing products of irradiated water, there would be no inhibition on irradiation of urease when the —SH groups were protected by *p*-chloromercuribenzoate. If inhibition were due to destruction or denaturation of the enzyme, it would occur even after conversion of the —SH groups to the R-S-Hg-benzoate. Urease was

TABLE X

Inhibition of Urease by Gamma Ray Irradiation
Protection with p-Chloromercuribenzoate

Urease, 1.7 micrograms in 1.1 cc. *p*-Cl-Hg-benzoate (*p*-Cl-Hg), 0.0001 M; glutathione (GSH), 0.01 M.

Dose	Enzyme	Enzyme + *p*-Cl-Hg	Enzyme + GSH	Enzyme + Hg + GSH
r	*units*	*units*	*units*	*units*
0	1,492	0	1,538	1,498
200	989	0	939	1,466

irradiated with 200 r of gamma rays in the presence of glutathione (0.001 M) and in the presence of *p*-Cl-Hg-benzoate (0.0001 M). Urease with glutathione was inhibited to the same extent as the enzyme alone. When to the irradiated enzyme containing *p*-Cl-Hg-benzoate there was added glutathione, the enzyme activity was restored completely (Table X). Protection of the —SH groups by formation of the reversible mercaptide compound protected the enzyme from the inhibiting action of gamma rays.

The Ionic Yields of Enzyme Inhibition by Ionizing Radiations.—There is little information on the relative efficiency of different radiations regarding chemical effects. In reactions in the gaseous state, the ionic yields with different ionizing radiations are in general similar. In the production of H_2O_2 on irradiation of oxygenated water, Frilley (8) reports similar ionic yields for x-ray and for alpha ray irradiation. Lanning and Lind (11) found that fairly strong solutions of HBr, HI, and $KMnO_4$ were decomposed by alpha rays with an ionic efficiency of the order of unity. Irradiation of tyrosine by alpha rays seems, however, far less efficient than by x-rays, according to Nurnberger (12). On irradiation of carboxypeptidase with alpha rays (irradiation with radon) and

with x-rays, Dale, Meredith, and Gray[2] found that the efficiency of alpha rays was only 5 to 9 per cent that of x-rays. Numerous biological effects have been measured simultaneously with x-rays and with gamma rays, such as the inhibition of mitosis in tissue cultures (13), the lethal action on *Drosophila* eggs (14), on *Drosophila* pupa (15), on mouse tumors (16). In all cases, the efficiency of gamma rays was 50 to 20 per cent less than that of x-rays.

Calculation of the ionic efficiency of alpha, beta, and x-rays on inhibition of crystalline phosphoglyceraldehyde dehydrogenase has shown that all three ionizing radiations had about the same efficiency, namely unity (Table XI). With gamma rays, the ionic yield was 0.7.

TABLE XI

Ionic Yields of Enzyme Inhibition by X-, Alpha, Beta, and Gamma Rays

Enzyme: Phosphoglyceraldehyde dehydrogenase (70 micrograms per cc., except in gamma rays where 14 micrograms were used). *M*, number of enzyme molecules inhibited; *N*, number of ion pairs produced on ionization of 1 cc. of water, assuming that x-rays produce 1.616×10^{12}; alpha rays, 1.90×10^{12}; beta rays, 1.8×10^{12}; and gamma rays, 1.79×10^{12}.

Ionizing radiation	Dose	*M*	*N*	Ionic yield
	r			
X-rays	200	3.01×10^{14}	3.23×10^{14}	0.93
Alpha rays	180	3.49×10^{14}	3.42×10^{14}	1.0
Beta rays	56.5	1.93×10^{14}	1.02×10^{14}	1.9
Gamma rays	50	6.51×10^{13}	9.5×10^{13}	0.7

DISCUSSION

The experiments presented here on the inhibition of the sulfhydryl enzymes, phosphoglyceraldehyde dehydrogenase and urease, by alpha, beta, and gamma rays, and reactivation (in the case of alpha rays) on addition of glutathione, are presented as further evidence that ionizing radiations inhibit sulfhydryl enzymes by oxidation of the —SH groups essential for enzyme activity. This specific action on the —SH groups was clearly shown in the urease experiments and irradiation with gamma rays. A dose of gamma radiation that inhibited the enzyme containing the —SH groups intact had no effect at all when the —SH groups were withdrawn from oxidation by their transformation into mercaptides. In fact, complete reactivation of the enzyme was obtained on addition of glutathione.

The rôle of H_2O_2 in the inhibition of sulfhydryl enzymes by ionizing radiations was shown by the partial protection produced on addition of small amounts of

[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.

catalase. This inhibiting action of H_2O_2 is probably restricted to oxidation of —SH groups. On irradiation with alpha rays, oxidation by H_2O_2 contributed 30 per cent of the total inhibition, while with beta rays there seemed to be a greater contribution.

The equal efficiency of alpha rays and x-rays in the inhibition of sulfhydryl enzymes, as contrasted with the greatly diminished efficiency of alpha rays in the inhibition of carboxypeptidase, is probably due to the different mechanisms of action. The former are inhibited by oxidation of the —SH groups, while carboxypeptidase inhibition seems to be due to protein denaturation (the mechanism of carboxypeptidase action is unknown). All ionizing radiations had the same efficiency in inhibiting phosphoglyceraldehyde dehydrogenase.

Ionizing radiations have two different actions on proteins: oxidation of their —SH groups—a reversible phenomenon—and denaturation and destruction of the molecule, an irreversible phenomenon. The first requires fewer ionizing radiations than the second. These observations become of considerable biological significance when they are considered together with the distribution of sulfhydryl groups in living cells. In fact, it has been shown by a number of investigators (see Brachet (17)) that an abundance of sulfhydryl compounds are required by cells in mitosis and in division and growth. In all probability, these sulfhydryl groups (which are different from the sulfhydryl groups of enzymes) are oxidized on irradiation of cells, and inhibition of mitosis and of cell division by ionizing radiations may be due to this oxidation. Since oxidation of sulfhydryl groups is in general a reversible process, the effects of small amounts of ionizing radiations might also be reversible.

SUMMARY

The activity of crystalline phosphoglyceraldehyde dehydrogenase and urease was decreased when dilute solutions of these sulfhydryl enzymes were irradiated with small doses of alpha rays from Po, beta rays from Sr^{89}, and gamma rays from Ra. Partial reactivation of the enzyme by addition of glutathione was obtained after inhibition with alpha rays. Evidence that these inhibitions are due to oxidation of the —SH groups of the enzymes was given by the irradiation of the mercury-mercaptide urease with gamma rays. This irradiated complex was completely reactivated by glutathione as was the non-irradiated enzyme. The ionic efficiency of all these ionizing radiations on inhibition of phosphoglyceraldehyde dehydrogenase was similar (ionic yield around 1).

The sulfhydryl groups of crystalline phosphoglyceraldehyde dehydrogenase were titrated by enzyme activity measurements and by ferricyanide oxidation.

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