THE EFFECT OF X-RADIATION ON THE GLUTATHIONE METABOLISM OF INTACT ERYTHROCYTES IN VITRO*

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ABSTRACT

The x-irradiation of intact washed erythrocytes results in an inhibition of the glyoxalase activity of the cells chiefly as a result of a decrease in the reduced glutathione level. The percentage inhibition is markedly increased by an increase in the dilution of the cells in physiological saline suggesting that the effect of radiation is indirect, *via* the production in the aqueous medium of free radicals, H_2O_2, etc. This is supported by the decrease in the inhibition produced by lowering the oxygen tension or by the addition of catalase. The inhibition of glyoxalase activity is also decreased by the addition of methylglyoxal, plasma, adenosine, inosine, glucose, and a number of other sugars to the erythrocyte suspension prior to radiation. Furthermore, some reactivation of the glyoxalase system results from the addition of plasma, glucose, adenosine, and inosine following radiation. These results are discussed in relation to the role of SH compounds, particularly glutathione, in the toxicity of ionizing radiations.

The possible relationship of cellular sulfhydryl groups to the development of radiation injury has received wide attention in recent years, chiefly as a result of the investigations of Barron and coworkers (1–3). These investigators observed that a number of purified enzyme systems requiring intact sulfhydryl groups for optimum activity were inactivated by ionizing radiations with an ionic yield generally greater than that observed in the inactivation of enzymes not containing essential sulfhydryl groups (1, 2). Similarly, low molecular weight sulfhydryl compounds appeared to be oxidized with an exceptionally high ionic yield of over 3 (3). These observations, in conjunction with the established importance of sulfhydryl groups in many biological processes (see reference 4), suggested to Barron that the fundamental lesion produced by ionizing radiations *in vivo* may be the oxidation of SH[1] groups essential to the normal metabolism of the cell.

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[1] The following abbreviations were used: SH, sulfhydryl: GSH, reduced glutathione; GSSG, oxidized glutathione; TPN, triposphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Existing experimental data have not as yet established the importance of sulfhydryl oxidation in the development of toxicity following lethal doses of radiation to the intact animal (see reference 5). The inability of a number of investigators to demonstrate a decrease in SH groups, or an inhibition of SH systems immediately following radiation has been correlated with the fact that the number of SH groups present within the cell exceeds "by several degrees of magnitude" the number which could be oxidized by lethal doses of radiation (6). Since most cellular SH systems are normally in a reversible sulfhydryl disulfide equilibrium, it is also possible that the oxidative effect of radiation on certain SH systems is quickly masked by the rapid reduction of the oxidized form by the normally operative reductive systems.

The irradiation of an aqueous solution of glutathione has been found by Barron and Flood (3) to result in a decrease in the reduced glutathione level. Other investigators, however, have been unable to demonstrate a fall in the blood glutathione level immediately following whole body radiation (7, 8). Previous investigations have indicated that washed erythrocytes can be used for the study of the oxidation and reduction of glutathione in the cytoplasm of an intact cell (9, 10). In such a preparation, glutathione is easily oxidized by molecular oxygen as demonstrated by a fall in the glutathione level (9, 10), and by a decrease in the glyoxalase activity (11, 10). However, the effect of molecular oxygen is not observed in the presence of glucose or a number of other metabolites, suggesting that, under these conditions, the reduction of glutathione occurs at a rate sufficient to maintain glutathione in its reduced form (10). The glyoxalase activity of the cell serves as a very sensitive indicator of the level of reduced glutathione under these conditions.

Conditions are described in the present study in which an inhibition of glyoxalase activity and a decrease in the reduced glutathione level of intact erythrocytes were produced by x-radiation and evidence is presented which suggests the operation of the reversible oxidation-reduction system, GSH $\rightleftharpoons$ GSSG, under these conditions.

Methods

Heparinized blood was collected from normal human subjects. The erythrocytes were washed three times with 0.16 M NaCl, and suspended in 0.16 M NaCl to a volume approximately twice that of the packed cell volume. All subsequent dilutions were made with 0.16 M NaCl. All reagents were Fisher "certified" except those otherwise indicated. The solutions were made isotonic with NaCl.

The erythrocytes were irradiated within 24 hours of collection in pyrex test tubes with a G. E. maxitron x-ray machine operated at 250 kv. and 30 ma. with the added filtration of 0.5 mm. Cu and 1 mm. Al (hvl 1.4 mm. Cu). The source to target distance was 36 cm. which produced a dose rate in air of 250 r per minute. The dose rate was checked prior to each experiment with a Victoreen r meter.

The glyoxalase activity of the erythrocytes was determined as previously described

(12, 10) within 1 hour of the completion of radiation unless otherwise indicated. When the undiluted erythrocyte suspension was employed, 0.05 ml. was added for each determination. However, when the glyoxalase activity of a diluted erythrocyte suspension was estimated, the volume of the suspension added to each Warburg vessel was increased proportionate to the dilution. In this way, equivalent numbers of erythro-

TABLE I

Effect of X-Radiation on the Glyoxalase Activity, Degree of Hemolysis, and Hematocrit Value of Intact Erythrocytes

The main compartment of duplicate Warburg vessels contained 0.05 ml. of the erythrocyte preparation, 0.4 ml. of 0.2 M $NaHCO_3$, and 0.16 M NaCl to a final volume of 2.0 ml. The side arm contained 0.2 ml. of 1 per cent methylglyoxal (Bios Laboratories, Inc.). The contents were gassed with 5 per cent CO_2–95 per cent N_2 for 10 minutes and then the contents of the side arm emptied into the main vessel. After an open period of 2 minutes the stopcocks were closed and the evolution of CO_2 measured. Temperature 30°C. Rate of shaking 120 per minute.

Radiation	Storage	Hematocrit reading	Hemolysis prior to assay	Glyoxalase activity		Hemolysis following assay
				μl. CO_2/60 min.	Difference	
r	*days*	*per cent*	*per cent*		*per cent*	*per cent*
—	0	47.0	0	407		8
2,000	0	47.0	0	406	0	8
5,000	0	47.0	0	395	−3	8
10,000	0	47.0	0	377	−7	8
15,000	0	47.0	0	369	−9	8
20,000	0	47.0	0	357	−12	8
30,000	0	47.0	0	342	−16	8
—	1	47.5	1.0	414		8
20,000	1	48.0	1.5	353	−15	10
30,000	1	49.0	4.5	329	−21	15
—	3	47.5	1.0	415		8
20,000	3	54.0	2.5	344	−17	14
30,000	3	55.0	9.0	294	−29	25
—	6	49.0	2.0	323		15
20,000	6	55.0	11.5	223	−31	46.5
30,000	6	55.0	20.0	174	−46	73.0

cytes were employed in each determination. The degree of hemolysis was determined as previously described (10). The reduced glutathione level was estimated by the cyanide-nitroprusside method of Grunert and Phillips (13). The hematocrit reading was determined by centrifugation using 0.05 ml. graduated pipettes.

RESULTS

Washed erythrocytes were suspended in 0.16 M NaCl to a hematocrit reading of 47.0 per cent and exposed to x-rays as indicated in Table I. Under these conditions, x-radiation at doses up to 5000 r had no effect on the glyoxalase

activity, degree of hemolysis, or hematocrit reading of the cells. However, an increase in the radiation dose from 10,000 r to 30,000 r did result in a slight but definite inhibition of glyoxalase activity which was not accompanied by any change in the degree of hemolysis or in the hematocrit value. The glyoxalase activity of the irradiated cells was further decreased by storage at 4°C. for several days (Table I). However, the more rapid development of hemolysis on the storage of irradiated erythrocytes as compared with un-

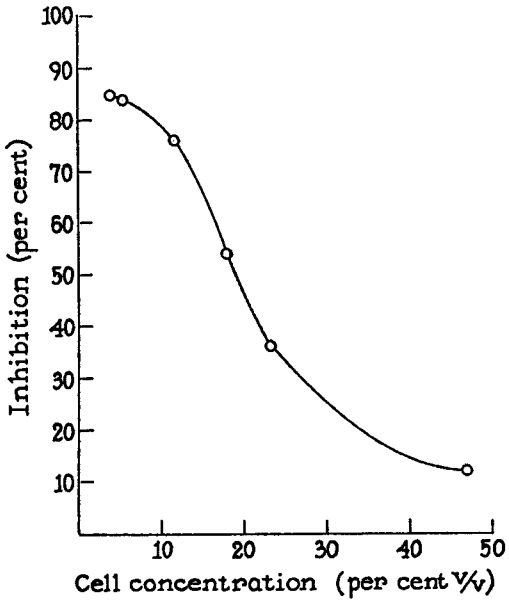

FIG. 1. The effect of the concentration of the erythrocyte suspension on the inhibition of the glyoxalase activity by x-radiation. The erythrocytes were diluted with 0.16 M NaCl to the concentrations indicated, irradiated with 20,000 r, and the glyoxalase activity determined.

treated cells (Table I) may account, to a large extent, for the more rapid decline in glyoxalase activity since hemolysis results in an almost complete loss of glyoxalase activity in the absence of added glutathione (11, 12). The increase in the hematocrit reading of the irradiated cells on storage (Table I) supports the suggestion that the development of hemolysis following x-radiation is due to swelling which results from an alteration in the osmotic equilibrium of the cells (14–16).

Dale (17) in his experiments on the effect of x-rays on purified enzymes in aqueous solution observed that the percentage inhibition by a given x-ray dose was increased by an increase in the dilution of the preparation. The

"dilution effect" as established earlier by Risse (18) and Fricke (19) on simpler compounds is a manifestation of the indirect action of ionizing radiations on solutes in aqueous solution through the production, in the solvent, of "activated molecules" (free radicals, H_2O_2, etc.). An effect of dilution on the response of intact cells to irradiation has been previously reported (20, 21). As can be seen in Fig. 1, the dilution of intact erythrocytes with 0.16 M NaCl prior to radiation resulted in a considerable increase in the percentage inhibition of the glyoxalase activity. Thus, the irradiation of a 3 per cent suspension (*v/v*) with 20,000 r produced an 85 per cent inhibition of the glyoxalase activity which fell exponentially with the radiation dose (Fig. 2).

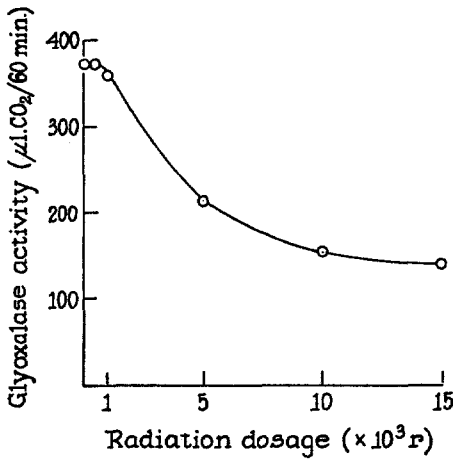

FIG. 2. The effect of the x-ray dose on the inhibition of the glyoxalase activity of a dilute suspension of erythrocytes. A 3 per cent suspension of erythrocytes was irradiated at the x-ray dose indicated and the glyoxalase activity determined.

The glyoxalase system consists of the enzyme components glyoxalase I and II and the cofactor, reduced glutathione (22, 23). As can be seen in Table II, the exposure of a 3 per cent suspension of erythrocytes to x-radiation at a dose sufficient to produce an inhibition of glyoxalase activity, also produced a fall in the reduced glutathione level of the cells as determined chemically. The role of glutathione in the inhibition of the glyoxalase activity by x-radiation was further emphasized by the following experiment. Aliquots of a 3 per cent suspension of erythrocytes, x-irradiated at doses up to 15,000 r, were completely hemolyzed by repeated freezing and thawing. Hemolysis of erythrocytes results in a fall in glyoxalase activity almost to zero, the activity being restored by the addition of glutathione (11, 12). The glyoxalase activity of the hemolyzed cells, to which was added a slight excess of non-irradiated glutathione, was compared with that of non-irradiated cells simi-

larly prepared. No significant difference in the glyoxalase activity of the irradiated and non-irradiated cells was observed, suggesting that the enzyme components of the glyoxalase system are relatively resistant to radiation under the conditions employed.

TABLE II

Effect of X-Radiation on the Glutathione Level of Erythrocytes

A 3 per cent erythrocyte suspension (*v/v*) was irradiated with x-rays at 500 to 15,000 r. The suspensions were centrifuged and the supernatant fluid was removed and 0.16 M NaCl was added to twice the packed cell volume. The figures for GSH are mg./100 ml. of cell suspension in saline (1:1, *v/v*).

Radiation	Reduced glutathione
r	*mg. per cent*
—	39.0
500	40.7
1000	42.5
5000	33.9
10000	30.2
15000	24.9

TABLE III

Effect of Oxygen on the Inhibition of Glyoxalase Activity by X-Radiation

Into the main vessel of duplicate Thunberg tubes was placed 2.0 ml. of a 3 per cent suspension of erythrocytes and into the side arm was placed 1.0 ml. of alkaline pyrogallol. The tubes were evacuated with a water pump and filled with commercial tank nitrogen. Tubes not containing alkaline pyrogallol were evacuated and filled with air. The tubes were shaken at room temperature for 20 minutes and x-irradiated at a dose of 10,000 r. The glyoxalase activity was determined over a 20 minute period and compared with that of erythrocytes similarly prepared but not irradiated.

Atmosphere	Inhibition
	per cent
Air	54
N_2	28

The effect of x-radiation on radiosensitive systems in aqueous solution is, in many instances, more pronounced in the presence of oxygen. Similarly in the present study, a decrease in the oxygen content of the erythrocyte suspension prior to radiation was found to result in a considerable decrease in the inhibition of the glyoxalase system (Table III). The addition of purified catalase (200 γ) to the suspension medium also resulted in a decrease in the inhibition, suggesting that H_2O_2 is involved to some extent. Although an increase in the catalase concentration produced a further protection of the glyoxalase system, crystalline bovine albumin was also protective at the

higher concentrations (Table IV). This suggests that the protective effect of catalase may be due in part to a non-specific protein protection.

TABLE IV

Effect of Added Catalase and Crystalline Bovine Albumin on the Inhibition of Glyoxalase Activity by X-Radiation

A 3 per cent suspension of erythrocytes was irradiated with 10,000 r in the presence or absence of catalase (Nutritional Biochemicals—lyophilized) or crystalline bovine albumin (Armour) in the amounts indicated. The glyoxalase activity was determined over a 20 minute period and compared with that of erythrocyte similarly diluted but not irradiated.

Supplement	Protection
	per cent
Catalase 200 γ	21
Catalase 600 γ	56
Catalase 800 γ	63
Albumin 200 γ	0
Albumin 600 γ	30
Albumin 800 γ	39

TABLE V

Effect of Methylglyoxal on the Inhibition of Glyoxalase Activity by X-Radiation

A 3 per cent suspension of erythrocytes was irradiated at 10,000 r in the presence of the supplements in the final concentrations indicated. The glyoxalase activity was determined and compared with that of non-irradiated cells similarly diluted.

Supplement	Glyoxalase activity (μl. CO_2/20 min.)		Inhibition
	Non-irradiated	Irradiated	
			per cent
—	129	52	60
Methylglyoxal (0.014 M)	18*	17*	
$NaHCO_3$ (0.02 M)	124	64	48
Methylglyoxal (0.014 M) + $NaHCO_3$ (0.02 M)	115	103	10

* Very severe hemolysis and clumping of cells.

Methylglyoxal, as the substrate for the glyoxalase system, combines with glutathione under the influence of the enzyme glyoxalase I to form an addition compound (22, 23). The inhibition of the glyoxalase system by x-radiation was very much decreased by the addition of methylglyoxal to the preparation just prior to radiation, provided that $NaHCO_3$ was added to a concentration sufficient to prevent the fall in pH resulting from the formation of lactic acid (Table V). In the absence of $NaHCO_3$, severe hemolysis and clumping of both the irradiated and non-irradiated cells occurred.

Plasma, glucose, fructose, mannose, adenosine, inosine, and to a lesser extent, maltose and galactose prevent to some extent the inhibition of the glyoxalase system of intact erythrocytes by oxygen (10). A similar effect of these substances on the inhibition of the glyoxalase system by x-radiation is indicated in Table VI and Fig. 3. Thus, the x-radiation of a 3 per cent suspension of erythrocytes with 10,000 r produced a 64 per cent inhibition of glyoxalase activity, which was completely prevented by the addition of

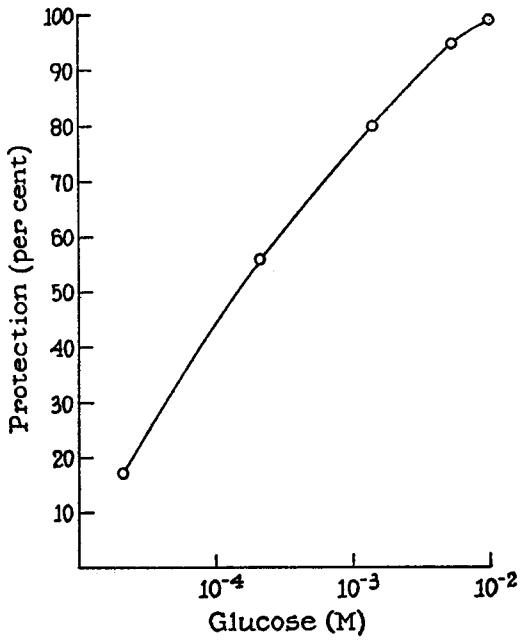

FIG. 3. The effect of glucose on the inhibition of glyoxalase activity by x-radiation. The experiment was conducted as in Table VI.

homologous plasma to a concentration of 45 per cent of the flask contents (Table VI). Similarly, glucose was protective in concentrations as low as 3.3×10^{-4}M, and produced complete protection at a concentration of 1×10^{-2}M (Fig. 3). A comparison of the protective effect of a number of sugars is shown in Table VI. The effect of adenosine and inosine is also indicated in Table VI.

The inhibition of glyoxalase activity produced by the x-radiation of a 3 per cent suspension of erythrocytes with 5,000 or 10,000 r was partially reversed by the addition of plasma, glucose, adenosine, or inosine to the erythrocyte preparation, immediately following radiation (Fig. 4). A slight increase in this effect was produced by increasing the period of incubation with the

TABLE VI

Effect of a Number of Substances on the Inhibition of the Glyoxalase Activity by X-Radiation

A 3 per cent suspension of erythrocytes was irradiated at a dose of 10,000 r in the presence or absence of the supplements in the final concentrations indicated. The glyoxalase activity (μl. CO_2/20 minutes) was compared with that of non-irradiated controls.

Supplement	Protection
	per cent
Plasma 10 per cent	75
Plasma 20 per cent	88
Plasma 45 per cent	100
Mannose 3.3×10^{-3} M	87
Fructose 3.3×10^{-3} M	77
Galactose 3.3×10^{-3} M	77
Maltose 3.3×10^{-3} M	77
Sucrose 3.3×10^{-3} M	66
Lactose 3.3×10^{-3} M	61
Adenosine 1×10^{-4} M	55
Adenosine 1×10^{-3} M	62
Adenosine 3×10^{-3} M	67
Inosine 1×10^{-4} M	58
Inosine 1×10^{-3} M	67
Inosine 3×10^{-3} M	76

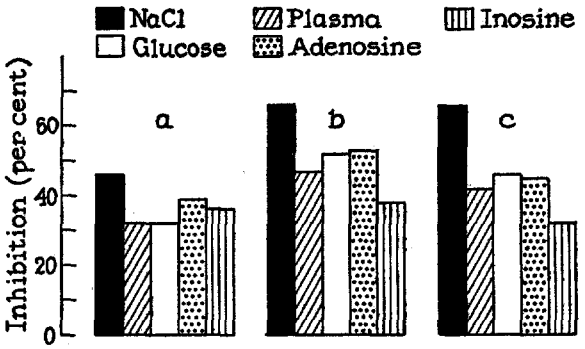

FIG. 4. The effect of plasma, glucose, adenosine, or inosine, added immediately following radiation on the inhibition of glyoxalase activity. A volume (0.5 ml.) of the erythrocyte preparation (hematocrit reading, 45) was added to a number of tubes containing 6.7 ml. of 0.16 M NaCl and the suspension was irradiated. Control tubes were similarly prepared but not irradiated. Within 5 minutes of the completion of radiation 0.8 ml. of 0.16 M NaCl, plasma, 0.167 M glucose, 0.01 M adenosine, or 0.01 M inosine was added to both the experimental and control tubes. The tubes were preincubated with the supplements at room temperature for the periods indicated and the glyoxalase activity determined. (*a*) radiation 5000 r, preincubation period 1 hour, (*b*) radiation 10,000 r, preincubation period 1 hour, (*c*) radiation 10,000 r, preincubation period 24 hours.

protective agents from 1 hour to 24 hours. A complete reversal of the inhibition was not observed under the conditions employed.

DISCUSSION

Barron and Flood (3) have observed that glutathione, in pure aqueous solution, is extremely sensitive to oxidation by ionizing radiations. The experiments described here indicate that x-radiation has a similar effect on glutathione in the intact erythrocyte as indicated by variations in glyoxalase activity. However, it should be emphasized that to demonstrate a marked effect on the glyoxalase system with moderate doses of x-radiation, well washed cells, considerably diluted with physiological saline, were required. Furthermore, plasma, and glucose in physiological concentrations largely prevent the inhibition of glyoxalase activity by x-radiation. This suggests that blood glutathione may be well protected from the oxidative effect of radiation *in situ*. This is consistent with the inability to demonstrate a fall in the blood glutathione level immediately following whole body radiation (7, 8).

The protective effect of glucose has been observed in the intact organism and in isolated radiosensitive systems, and is considered to be largely a result of the competitive acceptance of free radicals formed in aqueous solution (24, 25). The operation of such a mechanism in the present study is suggested by the protective effect of sugars (*i.e.* sucrose) which are not readily metabolized by the erythrocyte. Hollaender and Stapleton (26) have suggested that in some systems, the protection produced by glucose may be partly due to the hypoxia which results from the utilization of oxygen in the course of the metabolic degradation of the substrate. The largely anaerobic nature of the glucose metabolism of the erythrocyte would tend to minimize the importance of this mechanism in the present study. Another mechanism, however, may be responsible in part for the protective effect of glucose on the glyoxalase system. Barron and Flood (3) have suggested that x-radiation results chiefly in the oxidation of glutathione to its disulfide form. It has been observed that glucose and other metabolites are effective in maintaining the glutathione of washed erythrocytes in its reduced form in the presence of molecular oxygen (10) by the coupled oxidation of the metabolite with the reduction of oxidized glutathione by the glutathione reductase system (27). A similar oxidation-reduction system may also be operative during x-radiation as indicated below:

$$\text{Glucose etc.} \rightarrow \underset{\text{TPNH(DPNH)}}{\overset{\text{TPN(DPN)}}{\rightleftharpoons}} \overset{\text{GSH}}{\underset{\text{GSSG}}{\rightleftharpoons}} \leftarrow \text{X-radiation}$$

Glutathione reductase

Barron and coworkers (1, 2) have observed that sulfhydryl enzymes inactivated by low doses of radiation may be completely reactivated by the

addition of glutathione. It is, therefore, of interest that plasma, glucose, adenosine, and inosine produce a partial reactivation of the glyoxalase system (Fig. 4). This is compatible with the operation of the oxidation-reduction system depicted above and suggests that the maintenance of glutathione in its reduced form during radiation by normally operative reductive systems may be a factor in the protection of other sulfhydryl systems *in vivo*. It is of interest that a complete reactivation of the glyoxalase system was not observed under the conditions employed. This would suggest that glutathione may be affected by radiation in ways other than the simple oxidation to the disulfide form (28). The suggested importance of mixed disulfide formation in sulfhydryl-disulfide interactions, and the possible relationship of this phenomenon to the radioprotective properties of sulfhydryl and disulfide compounds (29–31) are of interest in this regard.

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