THE RELATIONSHIP OF HYDROGEN PEROXIDE TO THE INHIBITION OF THE GLYOXALASE ACTIVITY OF INTACT ERYTHROCYTES BY X-RADIATION*

By S. J. KLEBANOFF‡

(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada, and The Rockefeller Institute for Medical Research, New York)

(Received for publication, November 8, 1957)

ABSTRACT

The x-irradiation of a dilute suspension of erythrocytes results in a decrease in the glyoxalase activity of the cells as a result of a fall in the reduced glutathione level. The present paper deals with the possible role of H_2O_2 in this reaction.

The addition of intact erythrocytes to physiological saline previously irradiated with 150,000 r or 225,000 r results in a fall in the glyoxalase activity of the cells. The inhibition is prevented by the preincubation of the irradiated saline with catalase and is reversed by the addition of plasma, glucose, adenosine, and inosine to the cell suspension.

An inhibition of the glyoxalase activity is also produced by the addition of H_2O_2 to the suspension of erythrocytes. The inhibitory effect of H_2O_2 can be prevented and largely reversed by plasma, glucose, adenosine, and inosine. Methylglyoxal is also protective under these conditions.

Hydrogen peroxide formed continuously and in low concentrations by enzyme systems appears to be more effective than added H_2O_2 in inhibiting the glyoxalase system. The inhibition by H_2O_2–producing enzyme systems is minimized by the addition of catalase, plasma, glucose, methylglyoxal, and to a lesser extent, by adenosine and inosine, and is accentuated by the addition of sodium azide.

The results are discussed in relation to the role of H_2O_2 and catalase in the toxicity of ionizing radiations.

The formation of hydrogen peroxide by the irradiation of aqueous solutions has been observed repeatedly (1, 2) and the considerable influence of this substance on many biological systems has encouraged a great deal of speculation, and considerable experimentation towards elucidating the role, if any, of H_2O_2 in the toxicity of ionizing radiations. The glyoxalase activity of intact erythrocytes is inhibited by x-radiation largely as a result of a decrease in the reduced glutathione level of the cells (3). The increase in the per cent

* Supported in part by a grant from the Banting Research Foundation.

‡ Senior Fellow of the National Research Council of Canada.

Present address: The Rockefeller Institute for Medical Research, New York.

inhibition resulting from the dilution of the cells in physiological saline prior to radiation suggests that this effect is indirect, *via* the production of "activated molecules" in the aqueous medium. Because the concentration of a radiosensitive substance *within* an intact cell is unchanged by the dilution of the whole cell with a physiological salt solution, a "dilution effect" under these conditions would suggest that a large proportion of the effective activated molecules are formed in the extracellular fluid and that they pass to or through the cell membrane to react with the sensitive substance under investigation. Of the products of irradiated water, H_2O_2 is the most stable. That H_2O_2 is in part responsible for the inhibitory effect of x-radiation on the glyoxalase system is suggested by the partial protection afforded to the cells by the addition of catalase to the suspension medium (3). The purpose of the present investigation was to obtain further information on the role of H_2O_2 in the inhibition of the glyoxalase activity of intact erythrocytes of x-irradiation.

Methods

Source of Materials.—Human erythrocytes were collected and prepared as previously described (4). Special reagents were obtained commercially as follows: catalase (lyophilized), uricase, *d*-alanine, *d*-amino acid oxidase, glucose oxidase ("pure"), and sodium pyruvate from Nutritional Biochemicals Corporation; crystalline bovine plasma albumin from Armour Laboratories; hydrogen peroxide from Merck & Co. (superoxol 30 per cent); methylglyoxal (30 per cent solution) from K. & K. Laboratories; and gluconic acid-δ-lactone from California Foundation for Biochemical Research. All solutions were made isotonic with sodium chloride.

Procedures.—The x-irradiation of 0.16 M NaCl at doses of up to 20,000 r was performed as previously described (3). Irradiation at the higher dose levels was performed as follows: 250 kv.; 30 ma.; added filtration 0.5 mm. Al; source to target distance 13 cm. The dose rate as estimated with a Victoreen r meter was approximately 5000 r/minute.

The glyoxalase activity was determined as previously described (4, 5) with slight modifications as indicated below. In the investigation of the effect of H_2O_2 on the glyoxalase system, H_2O_2 was added to the erythrocyte suspension from a side arm after the contents of the vessel had been gassed with 5 per cent CO_2–95 per cent N_2. The stopcocks were then left open for 15 minutes to allow for the escape of oxygen formed by the degradation of hydrogen peroxide by cellular catalase. Methylglyoxal was then added, the stopcocks closed, and the glyoxalase activity determined. In the investigation of the effect of H_2O_2–generating enzyme systems, all components except methylglyoxal were added to the main compartment of the Warburg vessel. The reaction was initiated by the addition of the substrate (uric acid, glucose, *d*-alanine) to the flasks already containing all the components including the erythrocytes. The flasks were rapidly transferred to the bath and the contents incubated at 30°C. for the required period in an atmosphere of air. The flasks were then gassed with 5 per cent CO_2–95 per cent N_2. The removal of oxygen in this fashion minimized the formation of H_2O_2 by the enzyme systems. Methylglyoxal was added from the side arm and the gly-

oxalase activity determined. No gas exchange was observed in the absence of methylglyoxal.

The degree of hemolysis was determined after each estimation of glyoxalase activity as previously described (4).

RESULTS

Effect of X-Irradiated Saline

Taylor, Thomas, and Brown (6) were the first to note that the suspension medium previously irradiated may be toxic to intact cells. In their study, the irradiation of oxygenated 10 per cent yeast extract or water rendered the medium highly toxic or lethal to the protozoa, *Colpidium campylum*. The irradiation of the suspension medium was also found to reduce the survival time (7), delay the cleavage (7), and inhibit the respiration (8) of sea urchin spermatozoa, and to increase the mutation rate (9) and inhibit the growth (10) of bacteria. In some cases, H_2O_2 was demonstrated in the irradiated medium in concentrations sufficient to account for the observed toxicity (6, 7). When this was not found to be the case, the formation of organic peroxides was postulated (8, 9).

The effect of irradiated saline on the glyoxalase activity of intact erythrocytes is shown in Table I. A volume of 0.16 M NaCl was irradiated at a dose of 20,000 r and erythrocytes were added within 5 minutes of the completion of irradiation to form 3 to 24 per cent (*v/v*) suspensions. The x-irradiated saline was found to have no effect on the glyoxalase activity of the added erythrocytes under these conditions. In contrast, the irradiation of a dilute erythrocyte suspension with a similar x-ray dose results in a considerable inhibition of the glyoxalase activity of the cells (3). An increase in the radiation dose to 150,000 r or 225,000 r, however, did produce a substance in 0.16 M NaCl which was inhibitory to the glyoxalase system. The inhibition of glyoxalase activity was only slightly diminished (from 18 to 15 per cent) when the erythrocytes were added 24 hours after the irradiation of the suspension medium with 150,000 r, indicating that the toxic substance formed was relatively stable. That H_2O_2 is responsible for the toxic effect is suggested by the absence of organic material in the irradiated solution and by the complete prevention of the inhibition of glyoxalase activity by the preincubation of the irradiated solution with catalase for 1 hour at room temperature prior to the addition of the erythrocytes (Table II). This effect of catalase was destroyed by heating the enzyme for 15 minutes at 90°C.

It has been observed that the inhibition of the glyoxalase system by x-irradiation can be largely prevented and partially reversed by the addition of plasma, glucose, adenosine, and inosine (3). The inhibition of the glyoxalase system by x-irradiated saline can be completely reversed by the addition of these substances under the conditions described in Table III.

Effect of Hydrogen Peroxide

The effect of H_2O_2 on the glyoxalase activity of intact erythrocytes is shown in Fig. 1. Under the conditions employed, an inhibition was just evident at a H_2O_2 concentration of 1×10^{-4} M, whereas at a concentration of 5×10^{-3} M a 79 per cent inhibition was observed. The preincubation of the erythrocytes with H_2O_2 (final concentration 1×10^{-3} M) for 5, 15, 30, or 60 minutes prior to the addition of methylglyoxal did not alter the degree of inhibition. In no

TABLE I

Effect of X-Irradiated Saline

A volume of 0.16 M NaCl was irradiated as indicated below. The erythrocyte preparation (hematocrit value, 48) was added to the irradiated saline to produce the final concentration of erythrocytes indicated. After 30 minutes of incubation the glyoxalase activity of the suspension was determined and compared with that of erythrocytes similarly diluted but not irradiated. The volume of the erythrocyte suspension added to the Warburg vessel was increased proportionate to the dilution so that equal numbers of erythrocytes were employed in each determination.

Radiation	Concentration of RBC	Glyoxalase activity		Hemolysis
		μl. CO_2/20 min.	Difference	
r	*per cent v/v*		*per cent*	*per cent*
—	24	141		8
20,000	24	140	0	8
—	18	138		8
20,000	18	138	0	8
—	12	138		8
20,000	12	138	0	8
—	6	140		8
20,000	6	137	0	8
—	3	138		8
20,000	3	134	−3	8
—	3	147		7
150,000	3	121	−18	8
—	3	145		7
225,000	3	93	−36	7

instance could the decrease in glyoxalase activity be accounted for by an increase in hemolysis. The erythrocytes are rich in catalase, and the rapid degradation of H_2O_2 by this enzyme is indicated by a rapid evolution of oxygen associated with a change in the color of the erythrocytes from dark to bright red. The addition of methylglyoxal to the erythrocyte preparation prior to or immediately after the addition of H_2O_2 resulted in a considerable protection of the glyoxalase system (Table IV). The inhibition of the glyoxalase system by H_2O_2 was also prevented to a large extent by the addition of plasma, glucose, adenosine, and inosine (Table V). Of particular interest is the observation that these substances are able to reverse the inhibition of glyoxalase activity by

H_2O_2 to some extent (Fig. 2) as was the case with irradiated saline (Table III), and the x-irradiation of the erythrocyte suspension (3).

TABLE II

Effect of the Preincubation of Irradiated Saline with Catalase

A volume (7.1 ml.) of 0.16 M NaCl was irradiated with 150,000 r. Immediately after irradiation, 0.4 ml. of 0.16 M NaCl, 0.4 ml. of a 0.1 per cent solution of catalase, or 0.4 ml. of a 0.1 per cent solution of catalase heated for 15 minutes at 90°C. was added to both the irradiated saline and to an equal volume of non-irradiated saline and the solutions were preincubated for 60 minutes at room temperature. The erythrocytes were then added to produce a final concentration (*v/v*) of 3 per cent and the glyoxalase activity determined.

Supplement	Irradiation	Glyoxalase activity	
		μl. CO_2/20 min.	Inhibition
			per cent
NaCl	−	170	
Catalase	−	170	0
Catalase heated	−	167	2
NaCl	+	142	17
Catalase	+	171	0
Catalase heated	+	141	17

TABLE III

Effect of the Addition of Plasma, Glucose, Adenosine, and Inosine to Irradiated Saline

A volume of 0.16 M NaCl was irradiated as indicated below and the erythrocytes were added immediately after irradiation to produce a final concentration (*v/v*) of 3 per cent. The glyoxalase activity of the erythrocyte preparation was determined in the presence of the supplements at the final concentrations indicated.

Radiation	Supplements (Final concentration)	Inhibition
r		*per cent*
150,000	—	18
150,000	Plasma (10 per cent)	0
150,000	Glucose (3.3×10^{-3} M)	0
150,000	Adenosine (1×10^{-4} M)	0
150,000	Inosine (1×10^{-4} M)	0
225,000	—	36
225,000	Plasma (10 per cent)	3
225,000	Glucose (3.3×10^{-3} M)	9
225,000	Adenosine (1×10^{-4} M)	2
225,000	Inosine (1×10^{-4} M)	2

Effect of H_2O_2-Producing Enzyme Systems

A number of enzymes exist which catalyze the reduction of oxygen to hydrogen peroxide in association with the oxidation of the substrate. Hydrogen peroxide can be produced continuously for long periods in the presence of a

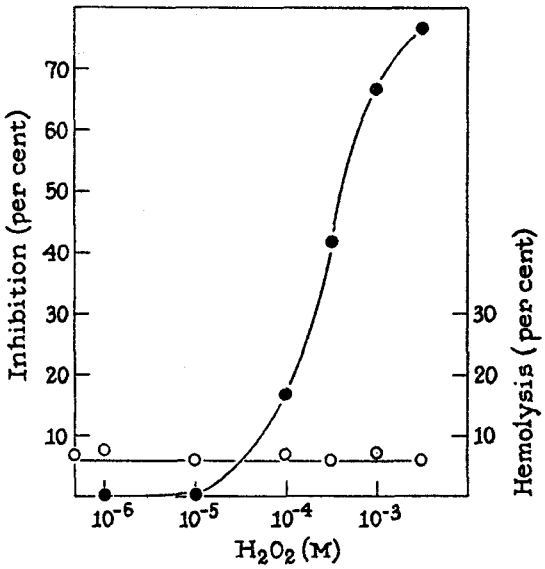

FIG. 1. Effect of H_2O_2 on the glyoxalase activity of intact erythrocytes. The main vessel contained 0.2 ml. of a 12.5 per cent suspension of erythrocytes, 0.4 ml. of 0.2 M $NaHCO_3$, and 0.16 M NaCl to a final volume of 2.0 ml. The first side arm contained 0.2 ml. of 0.16 M NaCl or 0.2 ml. of a H_2O_2 solution which on addition to the main vessel, formed the final concentrations indicated. The second side arm contained 0.2 ml. of 1 per cent methylglyoxal. The flasks were gassed with 5 per cent CO_2–95 per cent N_2. The contents of the first side arm were added and 15 minutes later, the contents of the second side arm were added. The evolution of CO_2 was measured for 20 minutes (filled circles, inhibition; open circles, hemolysis).

TABLE IV

Effect of Methylglyoxal

The main vessel contained 0.2 ml. of a 12.5 per cent suspension of erythrocytes, 0.4 ml. of 0.2 M $NaHCO_3$, and 0.16 M NaCl to a final volume of 2.0 ml. One side arm contained 0.2 ml. of 0.16 M NaCl or 0.2 ml. of 0.01 M H_2O_2. The second side arm contained 0.2 ml. of 1 per cent methylglyoxal. The flasks were gassed with CO_2–N_2 (5–95) and the contents of the side arms were emptied into the main vessel as indicated.

	Glyoxalase activity		Protection
	μl. CO_2/20 min.	Inhibition	
		per cent	*per cent*
No H_2O_2	152		
Methylglyoxal added 5 min. after H_2O_2	54	65	
Methylglyoxal added immediately after H_2O_2	85	44	32
Methylglyoxal added immediately prior to H_2O_2	129	15	77
Methylglyoxal added 5 min. prior to H_2O_2	136	11	83
Methylglyoxal added 10 min. prior to H_2O_2	138	10	85

low enzyme concentration relative to substrate concentration. The equilibrium set up between the formation of H_2O_2 in this fashion and its destruction by intracellular catalysts results in the presence of very low concentrations of H_2O_2 in contact with the erythrocytes throughout the experimental period. This is similar to the situation which results from the irradiation of an aqueous suspension of erythrocytes in the presence of oxygen, but is in contrast to that which results from the addition of H_2O_2 to the erythrocyte suspension or from the irradiation of the suspension medium prior to the addition of the erythrocytes. Under the latter conditions, the level of H_2O_2 is relatively high at the beginning of the incubation period with the erythrocytes and then falls precipitously, under the influence of cellular catalase. In the present

TABLE V

Protective Effect of Plasma, Glucose, Adenosine, and Inosine

The main vessel contained 0.2 ml. of a 12 per cent suspension of erythrocytes, 0.4 ml. of 0.2 M $NaHCO_3$, the supplements at the final concentrations indicated, and 0.16 M NaCl to a final volume of 2.0 ml. The first side arm contained 0.2 ml. of 0.16 M NaCl or 0.2 ml. of 0.01 M H_2O_2. The second side arm contained 0.2 ml. of 1 per cent methylglyoxal. The flasks were gassed with CO_2–N_2 (5–95). The contents of the first side arm were added and 15 minutes later, the contents of the second arm were added. The evolution of CO_2 was measured for 20 minutes.

Supplements	Inhibition
	per cent
None	66
Plasma 10 per cent	6
Glucose 1.7×10^{-2} M	6
Adenosine 1×10^{-3} M	3
Inosine 1×10^{-3} M	6

section the effect on the glyoxalase system of three H_2O_2-producing enzyme systems (uric acid–uricase; glucose–glucose oxidase; *d*-alanine-*d*-amino acid oxidase) was determined and compared to the effect produced by the addition of hydrogen peroxide.

(*a*) *Uric Acid–Uricase.*—Under the influence of uricase, uric acid is oxidized to allantoin while oxygen is reduced to hydrogen peroxide. The effect of the uric acid–uricase system on the glyoxalase activity of intact erythrocytes is shown in Table VI. Whereas uric acid alone appeared to have a slight inhibitory effect on the glyoxalase system, uric acid plus uricase produced a considerable inhibition. The effect of uricase was completely abolished by preheating the enzyme at 60°C. for 15 minutes. The erythrocytes were exposed to the uric acid–uricase system for periods varying from 5 to 120 minutes and the degree of inhibition determined. As can be seen in Fig. 3, the

inhibitory effect of the uric acid–uricase system increased with the incubation period. This is in contrast to the results observed with added H_2O_2.

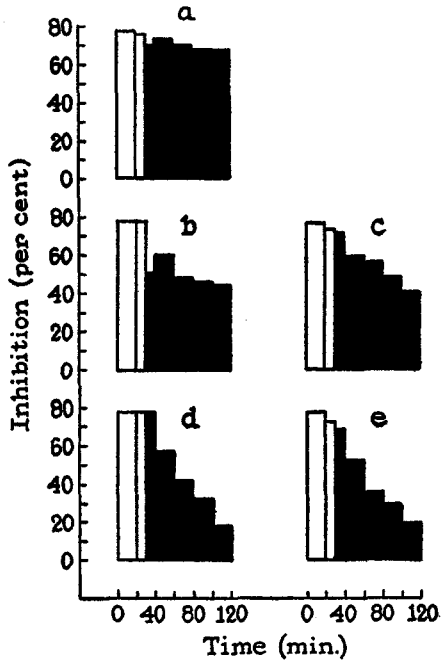

FIG. 2. Reversal of the H_2O_2-induced inhibition of glyoxalase activity. The main vessel contained 0.2 ml. of a 6 per cent suspension of erythrocytes, 0.4 ml. of 0.2 M $NaHCO_3$, and 0.16 M NaCl to a final volume of 2.0 ml. The number of erythrocytes per flask was half the number usually employed, to ensure continued activity for the 2 hour period of measurement. A volume (0.2 ml.) of 0.01 M H_2O_2 was added to the main vessel of the experimental flask but not to corresponding control flasks. One side arm contained 0.2 ml. of 1 per cent methylglyoxal which was added at zero time. The evolution of CO_2 by experimental flasks was compared with that of control flasks for a 30 minute period (open blocks). The contents of the second side arm ((*a*) 0.2 ml. of 0.16 M NaCl; (*b*) 0.2 ml. plasma; (*c*) 0.2 ml. of 0.17 M glucose; (*d*) 0.2 ml. of 0.01 M adenosine; (*e*) 0.2 ml. of 0.01 M inosine) were then added and the degree of inhibition determined (solid blocks).

Of the products of the oxidation of uric acid by uricase, allantoin at a final concentration of 10^{-3}M and 10^{-4}M was without effect on the glyoxalase system. The importance of H_2O_2 in the inhibitory effect of the uric acid–uricase system is emphasized by the protective effect of the addition of catalase to the suspension medium (Fig. 4). Crystalline bovine albumin in equivalent concentrations was without effect. Furthermore, sodium azide, a potent catalase inhibitor, considerably increased the inhibitory effect of the uric acid–uricase

TABLE VI

The Effect of the Uric Acid–Uricase System

The main vessel contained 0.4 ml. of 0.2 M $NaHCO_3$, 0.2 ml. of a 12.5 per cent suspension of erythrocytes, the supplements in the final concentrations indicated below, 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. The flasks were preincubated for 60 minutes at 30°C., gassed with CO_2–N_2 (5–95) for 10 minutes, and the glyoxalase activity determined.

Supplements	Glyoxalase activity	
	μl. CO_2/20 min.	Inhibition
		per cent
None	151	
Uric acid (5×10^{-4} M)	132	13
Uricase (100 γ per ml.)	143	5
Uric acid (5×10^{-4} M) + uricase (100 γ per ml.)	49	67
Uric acid (5×10^{-4} M) + uricase (100 γ per ml.) heated at 60°C. for 15 min	135	11

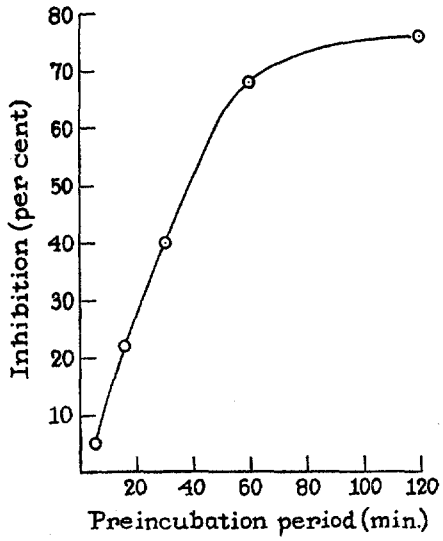

FIG. 3. Effect of the length of incubation on the inhibition by the uric acid–uricase system. The uric acid concentration was 5×10^{-4} M and the uricase concentration 100 γ per ml. The glyoxalase activity (μl. CO_2/20 minutes) was determined as in Table VI after preincubation periods of varying length.

system both in the presence and in the absence of added catalase (Fig. 5). Similar results were observed with added H_2O_2. Under the conditions employed, sodium azide alone was slightly inhibitory to the glyoxalase system at the higher concentrations.

The inhibitory effect of x-irradiation (3) and of added H_2O_2 (Tables IV and V) on the glyoxalase system was largely prevented by the addition of plasma, glucose, adenosine, inosine, and methylglyoxal. As can be seen in Fig. 6, plasma, glucose, and methylglyoxal largely prevented the inhibition by the uric acid–uricase system whereas a lesser protection was produced by adenosine and inosine under these conditions. It should be noted that erythrocytes incubated with plasma, glucose, adenosine, and inosine in the absence of the uric acid–uricase system have a greater glyoxalase activity than the

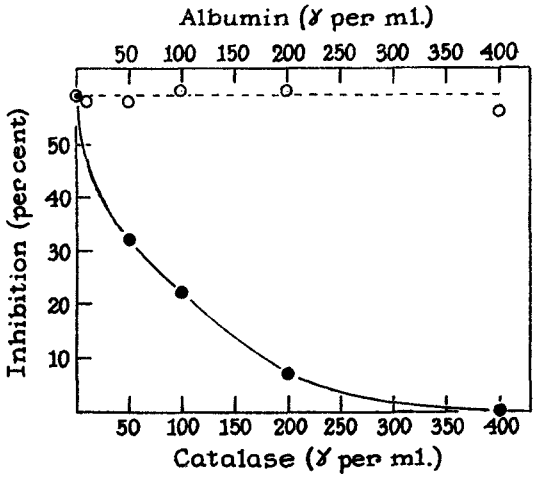

FIG. 4. Effect of catalase on the inhibition by the uric acid–uricase system. The main vessel of the experimental flasks contained 0.4 ml. of 0.2 M $NaHCO_3$, 0.2 ml. of a 12 per cent suspension of erythrocytes, 0.2 ml. of 1×10^{-3} M uric acid, 0.2 ml. of a 0.1 per cent solution of uricase, catalase (filled circles), and albumin (open circles) at the final concentrations indicated, 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. After an incubation period of 60 minutes, the glyoxalase activity (μl. CO_2/20 minutes) was determined and compared to that of control flasks which did not contain uric acid or uricase.

non-supplemented cells. This is probably due to the prevention by these substances of the over-all oxidation of glutathione by molecular oxygen (4) during the incubation period.

One molecule of H_2O_2 is formed for every molecule of uric acid oxidized by uricase. Thus, the maximum concentration of H_2O_2 which could be produced by the uric acid–uricase system is equivalent to the concentration of uric acid employed. A comparison of the inhibitory effect of H_2O_2 produced by the uric acid–uricase system with H_2O_2 added in concentrations equivalent to the uric acid concentration is shown in Fig. 7. The results indicate that H_2O_2 produced by the uric acid–uricase system is very much more effective

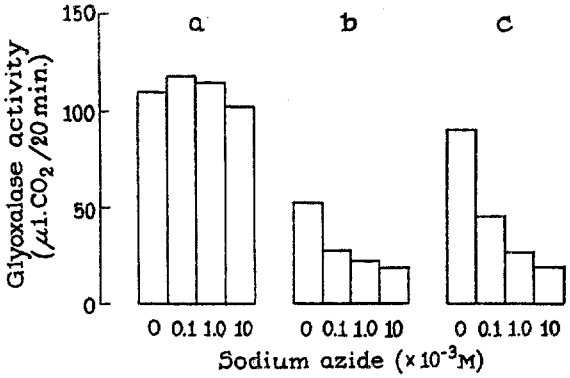

FIG. 5. Effect of sodium azide on the inhibition by the uric acid–uricase system. The main vessel contained 0.4 ml. of 0.2 M $NaHCO_3$, 0.2 ml. of a 12.5 per cent suspension of erythrocytes; sodium azide at the final concentrations indicated and 0.16 M NaCl to a final volume of 2.0 ml. Group (*a*) had no further additions; group (*b*), 0.2 ml. of 1×10^{-3} M uric acid and 0.2 ml. of a 0.1 per cent solution of uricase; group (*c*), as for group (*b*) plus 0.2 ml. of a 0.1 per cent solution of catalase. Incubation period 60 minutes.

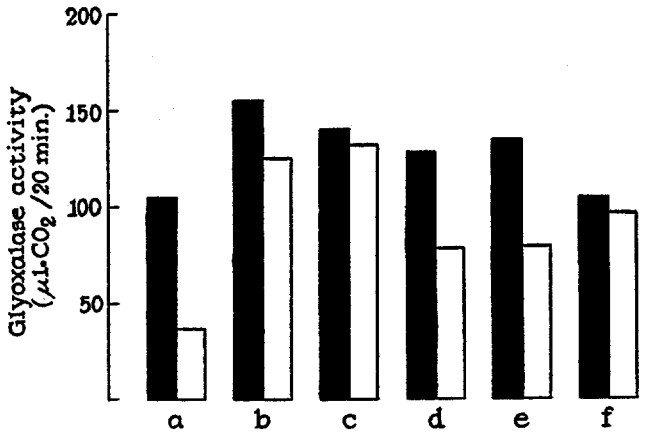

FIG. 6. Effect of plasma, glucose, adenosine, inosine, and methylglyoxal on the inhibition by the uric acid–uricase system. All flasks contained 0.4 ml. of 0.2 M $NaHCO_3$, 0.2 ml. of a 12.5 per cent suspension of erythrocytes, the supplements as indicated below, and 0.16 M NaCl to a final volume of 2.0 ml. Solid blocks represent control flasks in each group, whereas the open blocks represent flasks to which had been added 0.2 ml. of 1×10^{-3} M uric acid and 0.2 ml. of a 0.1 per cent solution of uricase. Group (*a*), no further addition; group (*b*), 0.2 ml. of plasma; group (*c*), 0.2 ml. of 0.01 M glucose; group (*d*), 0.2 ml. of 0.01 M adenosine; group (*e*), 0.2 ml. of 0.01 M inosine; group (*f*), 0.2 ml. of 1 per cent methylglyoxal. Incubation period 60 minutes.

than added H_2O_2 in inhibiting the glyoxalase activity of intact erythrocytes. A 60 minute incubation period was used. As can be seen in Fig. 3, maximum

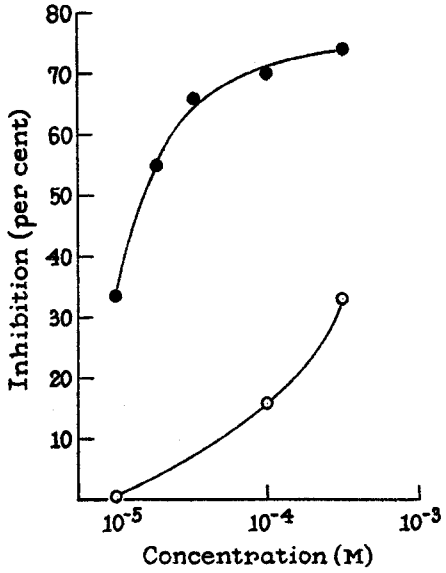

FIG. 7. A comparison of the inhibition produced by the uric acid–uricase system with that of added H_2O_2. To flasks containing 0.4 ml. of 0.2 M $NaHCO_3$, 0.2 ml. of a 12.5 per cent suspension of erythrocytes, and 0.16 M NaCl to a final volume of 2.0 ml. was added uric acid + uricase (filled circles) or H_2O_2 (open circles) in the final concentrations indicated and the glyoxalase activity (μl. CO_2/20 min.) compared to that of control flasks containing no additions. Incubation period 60 minutes.

TABLE VII

The Effect of the Glucose–Glucose Oxidase System

As for Table VI. Preincubation period 60 minutes.

Glucose, 5×10^{-4} M	−	+	+	+	+	+	+	+	+
Glucose oxidase, γ/ml.	−	−	5	10	50	100	300	500	−
Glucose oxidase, γ/ml., heated	−	−	−	−	−	−	−	−	100
Glyoxalase activity, μl. CO_2/20 min.	99	133	134	128	96	71	73	72	135

inhibition by the uric acid–uricase system may not have been attained in all instances.

(*b*) *Glucose–Glucose Oxidase System.*—In the oxidation of one molecule of glucose to gluconic acid–δ-lactone by the glucose oxidase (notatin) system, one molecule of H_2O_2 is formed. This reaction is of particular interest because glucose is also effective in maintaining glutathione in its reduced form in the presence of H_2O_2 as an oxidant. As can be seen in Table VII, the glyoxalase

activity of the erythrocyte preparation was greater after a 60 minute incubation period in the presence of glucose than in its absence. However, the addition of glucose oxidase to flasks containing glucose caused a fall in glyoxalase activity to levels below that of the unsupplemented control. The effect of glucose oxidase was completely abolished by heating the enzyme at 85°C. for 15 minutes. Glucose oxidase in the absence of glucose, or gluconic acid–δ-lactone was without effect.

TABLE VIII

Factors Affecting the Inhibition of Glyoxalase Activity by the Glucose–Glucose Oxidase System

As for Table VI. Preincubation period 60 minutes.

Glucose, × 10^{-3} *M*	−	2	2	2	2	2	2	2	2	−	2	1	0.1
Glucose oxidase, *150 γ/ml*	−	−	−	+	+	+	+	+	+	−	−	−	−
Sodium azide, *1* × 10^{-3} *M*	−	−	+	−	+	−	−	−	+	−	−	−	−
Catalase, *γ/ml*	−	−	−	−	−	200	400	800	400	−	−	−	−
H_2O_2, *2* × 10^{-3} *M*	−	−	−	−	−	−	−	−	−	+	+	+	+
Glyoxalase activity, *μl.* CO_2/ *20 min.*	101	125	124	78	30	110	123	126	45	48	125	129	119

TABLE IX

The Effect of the d-Alanine-d-Amino Acid Oxidase System

As for Table VI, except that the preincubation period was 90 minutes.

d-Alanine, *5* × 10^{-4} *M*	+	−	+	+	+	+	+
d-Amino acid oxidase, *1 mg./ml.*	−	+	+	−	+	+	+
d-Amino acid oxidase, *1 mg./ml.*, heated 60°C. for 15 min.	−	−	−	+	−	−	−
Catalase, *400 γ/ml.*	−	−	−	−	+	−	−
Catalase, *400 γ/ml.*, heated 90°C. for 15 min.	−	−	−	−	−	+	−
Albumin, *400 γ/ml.*	−	−	−	−	−	−	+
Inhibition, *per cent*	0	0	52	2	2	48	52

The inhibitory effect of the glucose–glucose oxidase system was completely abolished by catalase under the conditions described in Table VIII. The glyoxalase activity, under these conditions, was as high as in the presence of glucose alone, suggesting that the glucose concentration was not decreased by glucose oxidase to suboptimal levels. Crystalline bovine albumin in equivalent concentrations or catalase heated to 85°C. for 15 minutes was without effect. Sodium azide at a concentration of 10^{-3}M considerably increased the inhibitory effect of the glucose–glucose oxidase system and largely prevented the protective effect of added catalase (Table VIII). Sodium azide, at this concentration, had no effect on the glyoxalase system in the presence of glucose alone.

Hydrogen peroxide at a concentration equivalent to that of glucose produced a greater inhibition of glyoxalase activity than did the glucose–glucose oxidase system (Table VIII). However, under the conditions employed, the inhibition by the glucose–glucose oxidase system occurred in the presence of an excess of glucose as indicated by the effect of added catalase. As can be seen in Table VIII, glucose at a concentration $\frac{1}{20}$ of that employed in the glucose–glucose oxidase system completely abolished the inhibitory effect of added H_2O_2.

(*c*) *d-Alanine-d-Amino Acid Oxidase.*—As can be seen in Table IX, the inhibition of the glyoxalase activity of intact erythrocytes by the *d*-alanine-*d*-amino acid oxidase system was prevented by added catalase. Sodium pyruvate had no effect on the glyoxalase system under these conditions. A comparison of the effect of the *d*-alanine-*d*-amino acid oxidase system with that of added H_2O_2 is shown in Table X.

TABLE X

A Comparison of the Inhibition Produced by the d-Alanine-d-Amino Acid Oxidase System with That of Added H_2O_2

As for Table VI. Preincubation period 90 minutes.

d-Alanine, 5×10^{-4} *M*	−	+	+	+	+	−
d-Amino acid oxidase, *mg./ml.*	−	1.0	2.5	4.0	5.5	−
H_2O_2, 5×10^{-4} *M*	−	−	−	−	−	+
Glyoxalase activity *μl. CO_2/20 min*	121	80	55	42	41	81

DISCUSSION

Glutathione is readily oxidized to the disulfide form by H_2O_2 in the presence of a suitable catalyst (11, 12). The oxidation of glutathione results in a fall in the glyoxalase activity of intact erythrocytes (4). The inhibition of the glyoxalase activity by H_2O_2 can be prevented and largely reversed by the addition of plasma, glucose, adenosine, and inosine (Table V, Fig. 2), suggesting in conjunction with previous investigations (4, 3) that a reversible oxidation–reduction system may be operative as follows:—

$$\text{Glucose etc.} \rightarrow \left(\begin{matrix}\text{TPN(DPN)} \\ \text{TPNH(DPNH)}\end{matrix}\right) \left(\begin{matrix}\text{GSH} \\ \text{GSSG}\end{matrix}\right) \leftarrow H_2O_2$$

Glutathione reductase

The results obtained with H_2O_2 were qualitatively similar to those observed with x-irradiation (3).

The role of H_2O_2 in the toxicity of ionizing radiations is not clear. It is undoubtedly true that some of the manifestations observed in isolated radiosensitive systems are due to the formation of H_2O_2 in the solvent water. It

is equally true that some radiosensitive systems are insensitive to H_2O_2 and it appears that, in other instances, the amount of H_2O_2 which could be expected as a result of radiation is not sufficient to account for the complete toxicity. The data in the present paper would seem to place the glyoxalase system of intact erythrocytes in the last category. The concentration of H_2O_2 within the cell following x-irradiation is unknown. However, from measurements of the amount of H_2O_2 formed in biological fluids, it has been estimated that the H_2O_2 concentration might be in the order of 2.8×10^{-7}M per 1000 r (12). It is evident from the results presented in Fig. 1, that the addition of H_2O_2 to a suspension of erythrocytes in amounts equivalent to those expected on the irradiation of the suspension with x-ray doses up to 20,000 r had no effect on the glyoxalase activity of the cells despite the considerable inhibition produced by x-irradiation under similar conditions (3). An inhibition was observed only in the presence of a considerably higher concentration of H_2O_2. In the latter instance, the rapid degradation of H_2O_2 by the cell was evident. Similarly physiological saline, previously irradiated, was toxic to the glyoxalase system only when very high doses of radiation were employed.

Some caution, however, should be exercised in the interpretation of data obtained from the addition of H_2O_2 or from the irradiation of the suspension medium. Under these conditions, the H_2O_2 concentration in contact with the cells is maximal initially and decreases rapidly as a result of the presence of catalase within the cells. On the other hand, the irradiation of aqueous solutions in the presence of oxygen results in the production of H_2O_2 continuously and in very low concentrations throughout the period of irradiation It has been observed in the present study that H_2O_2 produced continuously and in low concentrations by a primary enzyme reaction inhibits the glyoxalase activity of intact erythrocytes and that the H_2O_2 so produced may be more effective than added H_2O_2 in this regard. This suggests that the role of H_2O_2 in the inhibition of the glyoxalase system, and perhaps other radiosensitive systems, by x-irradiation may be greater than is indicated by experiments involving the addition of H_2O_2 or the use of preirradiated saline.

The investigations of Keilin and Hartree (13–15), Chance (see reference 16), and others have demonstrated that catalase can catalyze the oxidation of a number of substances (hydrogen donors) by H_2O_2, in addition to causing the degradation of H_2O_2 to oxygen and water. A characteristic of the peroxidatic property of catalase which is of particular interest here is the very high efficiency of the oxidation of the hydrogen donor when H_2O_2 is generated continuously and in low concentrations by a primary oxidative reaction as compared to that observed when H_2O_2 is added to the reaction mixture in relatively high concentrations. Under conditions of low H_2O_2 concentration, the hydrogen donor is present in great excess and thus can successfully compete with H_2O_2 for oxidation by the primary catalase–H_2O_2 complex (16).

The destruction of H_2O_2 by catalase may serve to protect the organism from the toxicity of H_2O_2 during radiation regardless of whether the destruction occurs *via* the classical method of catalase action or by means of the peroxidatic oxidation of a secondary receptor. Indeed, it has been suggested that the protective effect of alcohol and sodium nitrite may be due to the accelerated degradation of H_2O_2 by catalase in the presence of those substances (17, 18). However, it is also possible that a radiosensitive substance of some importance to the cell may itself be oxidized by H_2O_2 in the presence of catalase. In this regard, there is some evidence to suggest that catalase may influence the oxidation of glutathione. Boeri and Bonnichsen (19) have reported that the oxidation of cysteine, glutathione, and thioglycollic acid by oxygen can be catalyzed by blood or liver catalase. Although H_2O_2 was not required for this reaction it should be borne in mind that H_2O_2 is formed on the oxidation of sulfhydryl compounds by molecular oxygen. Barron and Flood (20), however, have reported that the addition of catalase to an aqueous solution of glutathione decreases the oxidative effect of ionizing radiations on that substance. This suggests that under these conditions catalase does not promote the peroxidatic oxidation of glutathione. On the contrary catalase protects the glutathione molecule, presumably by the destruction of H_2O_2. There is also no evidence in the present paper to suggest that catalase is directly involved in the oxidation of glutathione by H_2O_2-forming enzyme systems. Thus sodium azide a potent catalase inhibitor, was found to increase the inhibitory effect of enzymatically produced H_2O_2 on the glyoxalase system. Sodium azide has also been found to significantly increase the mutagenic effect of ultraviolet radiation on microorganisms (21). However, a number of catalase inhibitors including sodium azide protect intact animals (22–25) and microorganisms (21) from the lethal effect of irradiation. These observations remain unexplained and their interpretation is complicated by the fact that these agents are not specific in their action on catalase and that sodium azide and hydroxylamine act also as hydrogen donors in the peroxidatic reaction stimulated by catalase (14). However, the possibility that, under certain conditions, catalase may be an active participant in the chain of reactions which lead from the primary ionization event to the resultant radiation injury bears further investigation.

I would like to acknowledge gratefully the continued interest and encouragement of Professor J. A. Dauphinee and the technical assistance of Mr. L. Zongor. I am also extremely grateful to Dr. D. G. Baker and Dr. C. G. Hunter of the Radiation Research Unit of the Banting and Best Institute of Medical Research for the irradiation of the saline.

BIBLIOGRAPHY

1. Risse, O., *Strahlentherapie*, 1929, **34**, 578.
2. Fricke, H., *Cold Spring Harbor Symp. Quant. Biol.*, 1934, **2**, 241.

3. Klebanoff, S. J., *J. Gen. Physiol.*, 1958, **41,** 725.
4. Klebanoff, S. J., *Biochem. J.*, 1957, **65,** 423.
5. Klebanoff, S. J., *Biochem. J.*, 1956, **64,** 425.
6. Taylor, C. V., Thomas, J. O., and Brown, M. G., *Physiol. Zool.*, 1933, **6,** 467.
7. Evans, T. C., *Biol. Bull.*, 1947, **92,** 99.
8. Barron, E. S. G., Flood, V. and Gasvoda, B., *Biol. Bull.*, 1949, **97,** 51.
9. Stone, W. S., Wyss, O., and Haas, F., *Proc. Nat. Acad. Sc.*, 1947, **33,** 59.
10. Wyss, O., Clark, J. B., Haas, F., and Stone, W. S., *J. Bact.*, 1948, **56,** 51.
11. Pirie, N. W., *Biochem. J.*, 1931, **25,** 1565.
12. Barron, E. S. G., Seki, L., and Johnson, P., *Arch. Biochem. and Biophysic.*, 1952, **41,** 188.
13. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, 1935–36, **119,** 141.
14. Keilin, D., and Hartree, E. F., *Biochem. J.*, 1945, **39,** 293.
15. Keilin, D., and Hartree, E. F., *Biochem. J.*, 1955, **60,** 310.
16. Chance, B., *in* The Enzymes, (J. B. Sumner and K. Myrback, editors), New York, Academic Press, Inc., 1951, **2,** pt. 1, 428.
17. Cole, L. J., and Ellis, M. E., *Am. J. Physiol.*, 1952, **170,** 724.
18. Cole, L. J., Bond, V. P., and Fishler, M. C., *Science*, 1952, **115,** 644.
19. Boeri, E., and Bonnichsen, R. K., *Acta Chem. Scand.*, 1952, **6,** 968.
20. Barron, E. S. G., and Flood, V., *J. Gen. Physiol.*, 1949–50, **33,** 229.
21. Berger, H., Haas, F. L., Wyss, O., and Stone, W. S., *J. Bact.*, 1953, **65,** 538.
22. Bacq, Z. M., and Herve, A., *Brit. J. Radiol.*, 1951, **24,** 617.
23. Boyland, E., and Gallico, E., *Brit. J. Cancer*, 1952, **6,** 160.
24. Feinstein, R. N., Cotter, G. J., and Hampton, M. M., *Am. J. Physiol.*, 1954, **177,** 156.
25. Feinstein, R. N., and Berliner, S., *Science*, 1957, **125,** 936.