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GLUTATHIONE-MEDIATED REDOX CYCLING OF ALLOXAN

MECHANISMS OF SUPEROXIDE DISMUTASE INHIBITION AND OF METAL-CATALYZED OH· FORMATION

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Abstract—The mechanism of the reaction between alloxan and GSH has been studied in the presence and absence of superoxide dismutase. Excess GSH reduced alloxan to dialuric acid, which underwent subsequent autoxidation, thus establishing a redox cycle in which O_2 and GSH in excess of the alloxan concentration were consumed. The major reaction products were H_2O_2 and GSSG. At each cycle, a small fraction of the alloxan reacted with GSH to form a 305 nm-absorbing adduct that gradually accumulated. In the presence of SOD, alloxan was reduced by GSH, but increasing concentrations of GSH progressively inhibited redox cycling as shown by decreased rates of O_2 uptake and GSH oxidation. With GSH:alloxan or dialuric acid molar ratios of >8–10:1, redox cycling was almost completely suppressed. A mechanism based on known reactions of GSH and dialuric acid is proposed. Alloxan and GSH, with an iron chelate present as catalyst, caused the hydroxylation of salicylate, an indicator of hydroxyl radical production. Hydroxylation was inhibited by catalase but not by superoxide dismutase, and it is attributed to the Fenton reaction in which the ferric catalyst is reduced by dialuric acid.

Alloxan (2,4,5,6[1H,3H]pyrimidinetetrone) is a potent diabetogenic agent in experimental animals, selectively destroying the β-cells of the pancreas [1]. It has been suggested [2, 3] that the cytotoxic action of alloxan is initiated by free radicals formed in a redox reaction between this substance and its reduction product, dialuric acid; autoxidation of the latter has been shown to generate O_2^-, H_2O_2, and, in the presence of a suitable catalyst, OH· [4]. The autoxidation of dialuric acid involves the intermediate formation of the alloxan radical and, as in the case of the closely-related pyrimidines divicine and isouramil [5, 6], the major oxidation pathway is an O_2^--dependent chain reaction, inhibited by superoxide dismutase (SOD). In the presence of SOD, an autocatalytic process involving interaction between dialuric acid and alloxan becomes important [6] while in the presence of a transition metal, a third oxidation mechanism, dependent upon H_2O_2, has been identified [4, 5].

While the autoxidation of dialuric acid has been extensively studied, the other half of the redox cycle, reduction of alloxan, has received comparatively little attention. One reducing agent which has been identified, however, is the ubiquitous tissue thiol, GSH [8]. This observation raises the interesting possibility that GSH, normally regarded as a cellular antioxidant, may *promote* the formation of oxidizing species by facilitating cycling between alloxan and dialuric acid. It has recently been shown, however, that in combination with SOD, GSH inhibits free-radical production from divicine and isouramil by maintaining the pyrimidines in their reduced forms [7]; the autoxidation of the structurally-similar dialuric acid would also be expected to be inhibited by GSH in the presence of SOD. Depending upon the circumstances, therefore, the interaction between GSH and alloxan could either be harmful or beneficial. These possibilities have been examined, and the results are described in the present report.

MATERIALS AND METHODS

Alloxan monohydrate was purchased from Sigma; dialuric acid was prepared by the method of Biltz and Damm [9]. Concentrations of the latter were determined using ε_{273} 16,000 l mol^{-1} cm^{-1} [10].

Ferrous ammonium sulphate (May & Baker) was of analytical grade. Chelex-100 was purchased from Bio-Rad Laboratories and was equilibrated to pH 7.0 before use: all other biochemicals were from Sigma.

Spectral changes during the interaction between alloxan or dialuric acid and GSH were monitored on a Pye-Unicam PU 8000 spectrophotometer. Reactions were conducted at 24° in 50 mM phosphate buffer, pH 7.4, containing 50 μM diethylenetriaminepenta-acetic acid (DPTA). In longer-term experiments on GSH oxidation and OH· formation, Chelex-treated 50 mM phosphate buffer, pH 7.0, was employed and the temperature was maintained at 25°. GSH was determined by the DTNB method [11] and GSSG by the method of Bernt and Bergmeyer [12]. Oxygen uptake was measured using a Yellow Springs Instruments Model 25 oxygen monitor.

Formation of OH· during the interaction between alloxan and glutathione was detected by the aromatic hydroxylation technique, a sensitive and specific assay system for this species [13]. Hydroxylation products of salicylate, formed by reaction of the

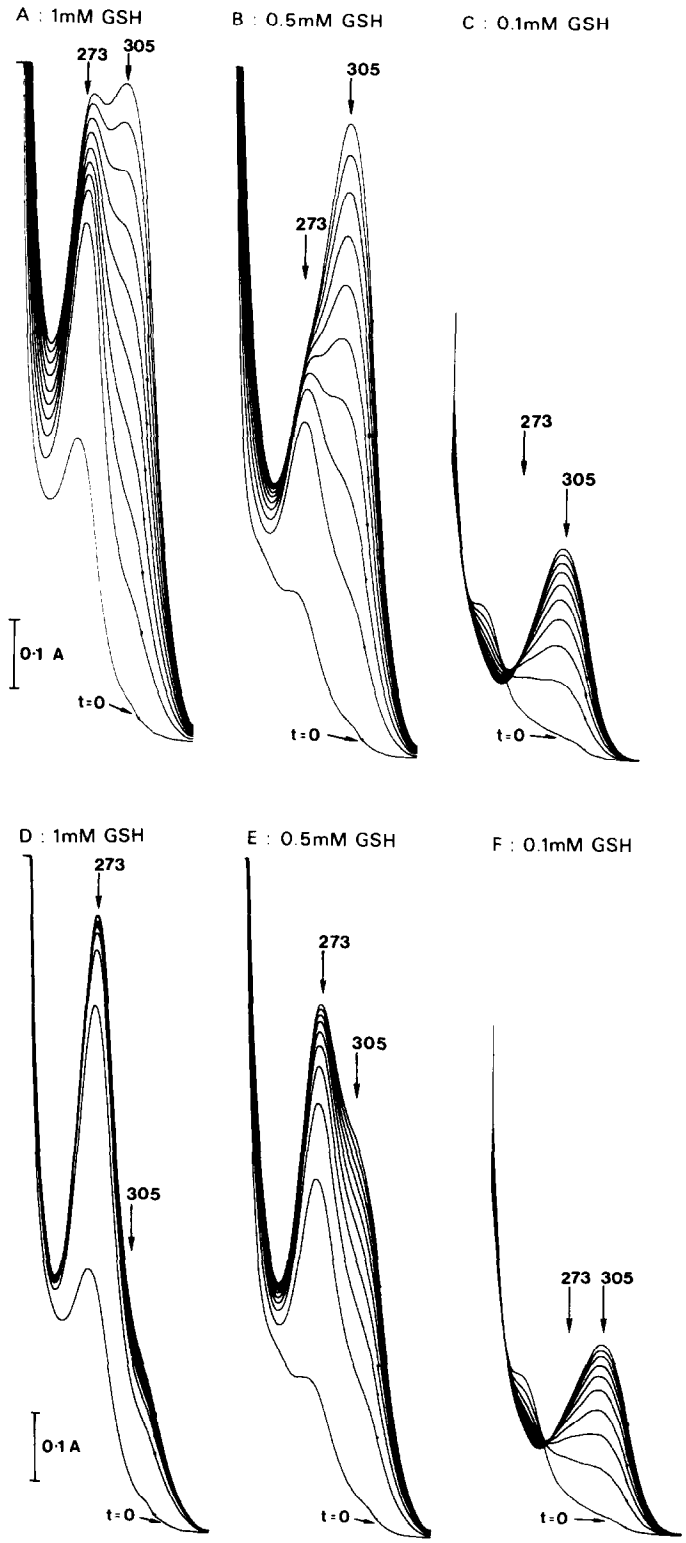

Fig. 1. Spectral changes during the interaction of alloxan with glutathione. A–C, no SOD; D–F, SOD (10 μg/ml) present. Solutions containing alloxan (50 μM) and DPTA (50 μM) were incubated at 24° in pH 7.4 phosphate buffer containing GSH at the concentrations indicated. Spectra between 360 and 240 nm were recorded at 2 min intervals starting immediately after adding alloxan to GSH.

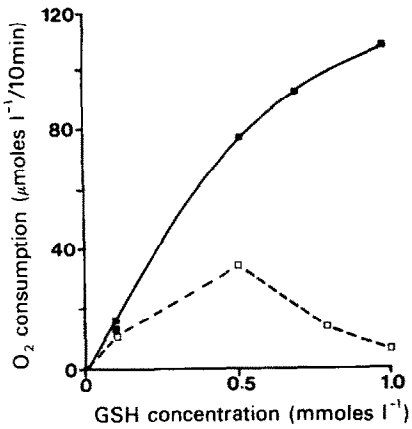

Fig. 2. Oxygen consumption during the interaction between alloxan and GSH. Oxygen uptake was measured under the same conditions as Fig. 1 except that the alloxan concentration was 55 μM. Solid line, no SOD; broken line, SOD 10 μg/ml.

latter with OH', were extracted and assayed colorimetrically [13], being quantified by reference to a standard curve prepared using 2,3-dihydroxybenzoic acid.

RESULTS

Interaction of alloxan with GSH: reduction to dialuric acid and formation of "Compound 305"

Addition of alloxan to a neutral solution of GSH at a molar ratio of 1 : 100 led to an immediate increase in absorption at 273 nm, characteristic of dialuric acid. The intensity of this peak with 50 μM alloxan and 5 mM GSH was 0.785 ± 0.036 absorbance units (mean ± SD, N = 5), indicating greater than 98% reduction; the spectrum remained unchanged for more than 30 min. At molar ratios of alloxan : GSH of 1 : 20 and 1 : 10, reduction to dialuric acid was still observed, but the spectrum changed with time, with a shoulder at 305 nm gradually changing into a peak at this wavelength (Fig. 1A–C). This peak has previously been identified [8] as due to formation of a glutathione-alloxan adduct ("Compound 305"), although the structure of this substance has not been determined. At still lower ratios of alloxan to GSH, no increase in A_{273} was recorded, and only the "Compound 305" was produced. SOD had little effect upon the reaction when the GSH : alloxan ratio was low, but as this ratio increased, SOD progressively stabilized dialuric acid and decreased the rate of formation of "Compound 305" (Fig. 1D–F).

Redox cycling during the interaction between alloxan and GSH

No O_2 consumption was recorded in buffered aqueous solutions of alloxan alone. In the presence of GSH, however, significant O_2 uptake was recorded, the rate of which increased with increasing concentration of the thiol (Fig. 2). The extent of O_2 consumption was in excess of the alloxan concentration, indicating that a cyclic process was occurring.

GSH was oxidized during its interaction with alloxan (Fig. 3) and the involvement of redox cycling was confirmed by the observation that 50 μmoles l^{-1} of alloxan destroyed 4.64 ± 0.04 mmoles l^{-1} of GSH over a 7 hr period. During the same time interval, 2.22 ± 0.13 mmoles l^{-1} of oxidized glutathione were formed, corresponding to a 96% conversion of GSH to GSSG.

Both O_2 uptake and GSH oxidation were strongly inhibited by SOD, particularly at higher GSH concentrations (Figs 2 and 3).

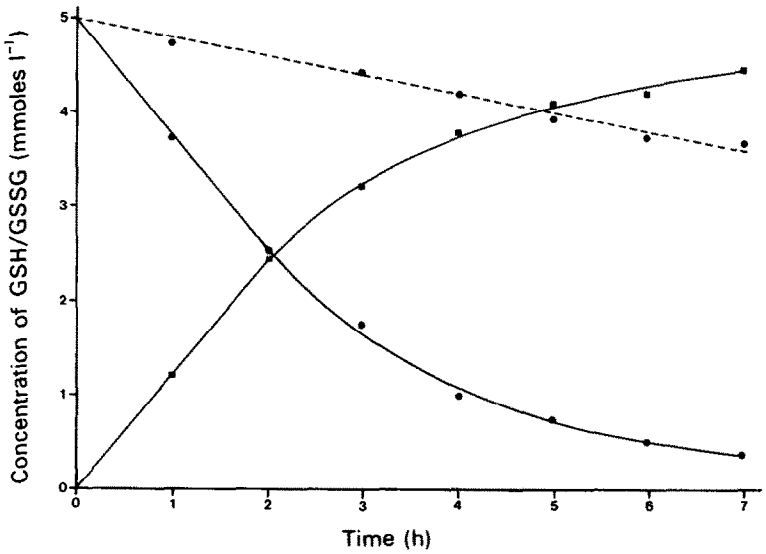

Fig. 3. Glutathione oxidation during the interaction between alloxan and GSH. GSH (5 mM) was incubated with alloxan (50 μM) at 25° in pH 7.0 phosphate buffer. ●, GSH concentration (solid line, no SOD; broken line, SOD 25 μg/ml); ■, GSSG concentration, expressed in terms of GSH equivalents, i.e. twice the measured GSSG concentrations.

Inhibition by SOD of coupled dialuric acid and GSH oxidation

Dialuric acid autoxidized rapidly in the absence of GSH, as indicated by a decrease in A_{273} (Fig. 4A). With increasing concentration of GSH, the rate of decrease in A_{273} gradually became less and there was concomitant formation of "Compound 305". With a GSH:dialuric acid molar ratio of 13:1 (Fig. 4A), there was an increase in A_{273}, due to overlap of the 305 nm band (cf. Fig. 1 for alloxan). SOD alone retarded dialuric acid oxidation (Fig. 4B), and combinations of SOD and GSH synergistically inhibited both the loss of A_{273} and formation of "Compound 305". At GSH:dialuric acid ratios >10:1, A_{273} increased slightly then remained constant for at least an hour, and there was only a small increase in A_{305} (Fig. 4B). Thus under these conditions there was very little oxidation of dialuric acid or adduct formation. None of the spectral changes was influenced by catalase.

In the absence of GSH, solutions of dialuric acid initially consumed O_2 rapidly, with a total O_2 consumption approximately equal to the initial dialuric acid concentration. GSH concentrations up to 400 μM did not alter initial rates of O_2 uptake (Fig. 5), but these rates were maintained for prolonged periods, indicating that redox cycling was occurring. In the presence of SOD, rates of O_2 uptake became constant after a short lag. Increasing concentrations of GSH progressively increased the lag time and decreased the maximum rate of O_2 uptake (Fig. 5). Thus with GSH:dialuric acid molar ratios in excess of 8:1, O_2 uptake was almost completely suppressed. Studies varying the dialuric acid concentration (not shown) showed that the ratio of the two compounds, rather than the concentration of GSH, was critical. O_2 uptake by this system could be stimulated by the addition of alloxan (not shown). Adding catalase at any time during the reaction led to recovery of 45–47% of the O_2 consumed (compared with 50% if it had all been converted to H_2O_2).

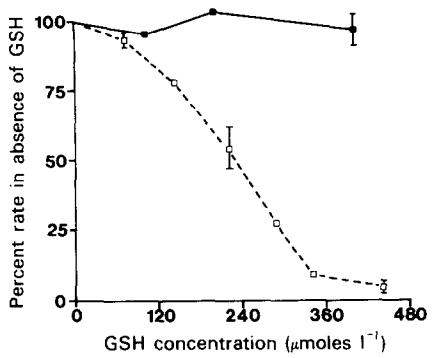

Fig. 5. Effect of SOD on oxygen uptake by dialuric acid in the presence of glutathione. Solutions containing dialuric acid (45 μM) and DPTA (50 μM) were incubated at 24° in pH 7.4 phosphate buffer containing various concentrations of GSH: ■——■, no SOD; □----□, SOD 10 μg/ml.

The time course of GSH oxidation paralleled that of O_2 consumption. Thus in the absence of SOD there was immediate rapid loss of GSH at all concentrations, whereas with SOD present loss of GSH became progressively less with increasing concentration (Fig. 6). Catalase had little effect on the loss of GSH, except in the latter stages when it was slightly inhibitory.

Cysteine was found to be as effective as GSH in prolonging O_2 uptake by dialuric acid, and the uptake was also inhibited by SOD (data not shown).

Formation of hydroxyl radical during the interaction of alloxan with GSH

Hydroxyl radical formation during the reaction of

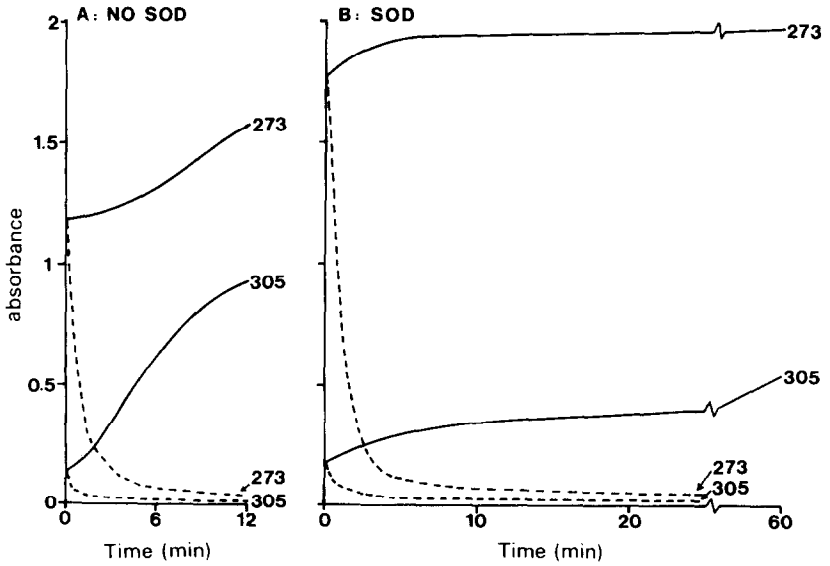

Fig. 4. Spectral changes during the interaction of dialuric acid with glutathione. Dialuric acid was incubated at 24° in pH 7.4 phosphate buffer containing 50 μM DPTA without GSH (broken lines) or in the presence of 1 mM GSH (solid lines). Absorbances at 273 and 305 nm were recorded at regular intervals: (A) no SOD, 75 μM dialuric acid; (B) SOD, 10 μg/ml, 110 μM dialuric acid.

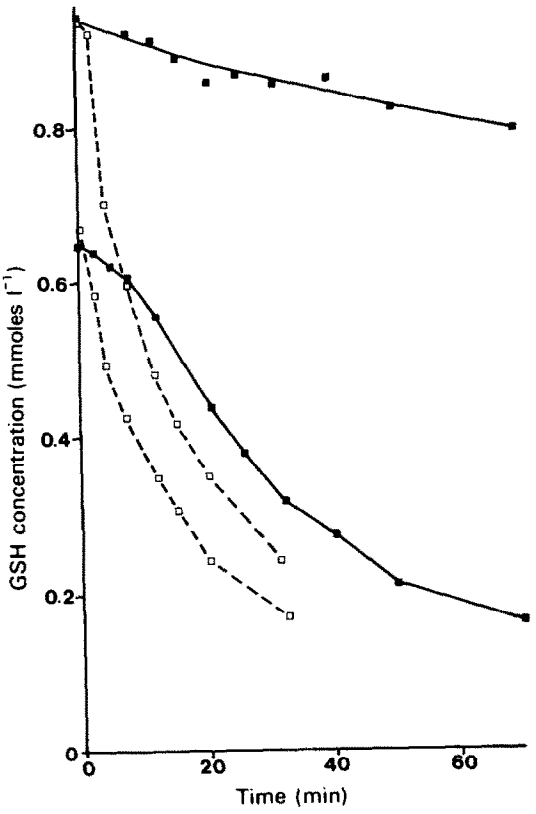

Fig. 6. Effect of SOD on GSH oxidation in the presence of dialuric acid. Solutions containing dialuric acid (80 μM), DPTA (50 μM) and either 0.65 or 0.94 mM GSH were incubated at 24° in pH 7.4 phosphate buffer: □-----□; no SOD; ■——■, SOD 10 μg/ml.

alloxan with glutathione was studied by the aromatic hydroxylation technique, which detects $OH^{\cdot}$ by virtue of its ability to oxidize salicylate to dihydroxybenzoate. Little hydroxylation occurred in the absence of added metal catalyst (Table 1). Significant $OH^{\cdot}$ production was recorded, however, in the presence of unchelated iron, iron–DTPA and, particularly, iron–EDTA. Iron-mediated $OH^{\cdot}$ production from alloxan and GSH was little affected by SOD but was almost completely abolished by catalase (Table 2).

Table 1. Hydroxyl radical formation during the interaction of alloxan with GSH

Addition	2,3-Dihydroxybenzoate formed (μmoles l^{-1})
None	17 ± 1
Fe	45 ± 1
Fe-EDTA	214 ± 7
Fe-DPTA	133 ± 3

GSH (5 mM) was incubated with alloxan (50 μM) in pH 7.0 phosphate buffer containing 2.5 mM potassium salicylate at 25° for 18 hr. After acidification, salicylate hydroxylation products were extracted and assayed. The concentration of iron (added as ferrous ammonium sulphate) was 100 μM; that of chelating agents, when added, was 110 μM. Results shown are the means and SD of 4 determinations.

DISCUSSION

In accord with an earlier report [8], alloxan was found to undergo two distinct reactions with GSH—reduction, to form dialuric acid, and conjugation, producing "Compound 305", of unknown structure. The reduction proceeds in two 1-electron steps (Reactions 1 and 2; A = alloxan, $AH^{\cdot}$ = alloxan radical, AH_2 = dialuric acid); the intermediate formation of the alloxan radical has been demonstrated by ESR [14].

$$A + GSH \rightarrow AH^{\cdot} + GS^{\cdot} \quad (1)$$

$$AH^{\cdot} + GSH \rightarrow AH_2 + GS^{\cdot} \quad (2)$$

$$A + GSH \rightarrow \text{"Compound 305"} \quad (3)$$

These reactions are in competition with the irreversible formation of the conjugate (Reaction 3), and the pathway followed in the reaction between alloxan and GSH was found to depend upon the molar ratios of the reactants.

At high ratios of GSH to alloxan, reduction was rapid and complete, indicating that Reactions 1 and 2 are faster than Reaction 3. Mass action considerations indicate that reduction would be particularly fast at high GSH concentrations, and alloxan would therefore be maintained in the reduced state. This explains the failure to observe "Compound 305" under these conditions as dialuric acid does not form a conjugate with GSH [8]. Continued regeneration of alloxan by redox cycling would allow a little "Compound 305" to form at each cycle, resulting in its gradual accumulation. This occurred at intermediate ratios of the reactants. The observed inhibition of "Compound 305" formation by SOD is consistent with this proposal, since SOD inhibits dialuric acid autoxidation, as discussed below.

The spectral data suggested that redox cycling was taking place during the interaction between alloxan and GSH, and this was confirmed in experiments showing O_2 uptake and cyclic oxidation of GSH in the presence of the pyrimidine. Such redox cycling requires that dialuric acid undergoes autoxidation in the presence of GSH.

In the absence of GSH and without a metal

Table 2. Effect of SOD and catalase on iron–EDTA mediated hydroxyl radical formation during the interaction of alloxan with GSH

Addition	2,3-Dihydroxybenzoate formed (μmoles l^{-1})
None	190 ± 10
SOD	161 ± 4 (−15%)
CAT	9 ± 2 (−95%)

Conditions were as described in the legend to Table 1, with addition of Fe^{II} (100 μM) and EDTA (110 μM). The concentration of each enzyme was 200 μg ml^{-1}, results shown are the means and SD of 4 determinations.

catalyst, dialuric acid autoxidation is initiated by reaction with molecular oxygen (Reaction 4) and propagated by a radical chain consisting of reactions 5 and 6 [6, 15]:

$$AH_2 + O_2 \rightarrow AH^{\cdot} + O_2^{\cdot-} + H^+ \quad (4)$$

$$AH^{\cdot} + O_2 \rightarrow A + O_2^{\cdot-} + H^+ \quad (5)$$

$$AH_2 + O_2^{\cdot-} + H^+ \rightarrow AH^{\cdot} + H_2O_2 \quad (6)$$

In the presence of GSH, reduction of the alloxan radical by Reaction 2 will compete with its reaction with oxygen, thereby inhibiting $O_2^{\cdot-}$ production via Reaction 5. However, the glutathionyl radical so formed is known to generate $O_2^{\cdot-}$ via Reactions 7 and 8 [16, 17]. GSH should not, therefore, affect production of $O_2^{\cdot-}$ from $AH^{\cdot}$ and the $O_2^{\cdot-}$-mediated chain oxidation of dialuric acid should continue.

$$GS^{\cdot} + GS^- \rightleftharpoons G\dot{S}\bar{S}G \quad (7)$$

$$G\dot{S}\bar{S}G + O_2 \rightarrow GSSG + O_2^{\cdot-} \quad (8)$$

In accord with observation, this mechanism predicts that O_2 uptake by GSH/alloxan would continue unabated even though dialuric acid was maintained in the reduced form; it is also consistent with the observed stoichiometry of GSH oxidation to GSSG.

The cyclic reaction between GSH and alloxan was inhibited by SOD, as reflected by both O_2 uptake and GSH oxidation. In accord with studies with divicine and isouramil [7], this effect is attributable to inhibition of dialuric acid autoxidation in the presence of GSH and SOD. Formation of "Compound 305" is not of significance in the inhibitory process, since a similar effect was recorded with cysteine, which does not form a comparable conjugate with alloxan.

Inhibition by SOD can be explained in terms of the proposed reaction mechanism. When SOD is present and Reaction 6 prevented, dialuric acid autoxidation still occurs but by an alternative radical chain sequence involving Reactions 5 and 9 [6].

$$A + AH_2 \rightleftharpoons 2AH^{\cdot} \quad (9)$$

Since this process requires the participation of alloxan, it is autocatalytic, and a characteristic lag in dialuric acid oxidation in the presence of SOD is observed [6]. GSH extended the lag as well as progressively decreasing the autoxidation rate to near zero. This can be explained by GSH competing with O_2 for $AH^{\cdot}$, thus decreasing the formation of alloxan in Reaction 5, and inhibiting the autocatalytic reaction. Although the thiyl radical is formed, $O_2^{\cdot-}$ resulting from its subsequent reactions will be destroyed by SOD, and chain reactions will be avoided. Oxidation will then occur only by the initiation process (Reaction 4) which is known to be slow [6]. Thus by preventing buildup of alloxan, GSH in combination with SOD is able to suppress redox cycling of dialuric acid. This mechanism is consistent with the observation that addition of alloxan to the dialuric acid/SOD/GSH system stimulated O_2 uptake; Reaction 9, forming $AH^{\cdot}$, would be followed by Reactions 2, 7 and 8, the last of which involves O_2 utilization. With alloxan as starting point, after initial reduction by GSH a mechanism similar to that with dialuric acid would operate.

A further mechanism for dialuric acid autoxidation involves a transition metal and H_2O_2, and under appropriate conditions, $OH^{\cdot}$ is formed [4]. Significant $OH^{\cdot}$ production was recorded during the interaction between GSH and alloxan in the presence of iron compounds. This metal is well-known as a promoter of $OH^{\cdot}$ production in systems generating "active oxygen" species, often operating through the iron-catalyzed Haber–Weiss reaction [18–20]:

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + OH^{\cdot} + OH^- \quad (10)$$

$$Fe^{III} + O_2^{\cdot-} \rightarrow Fe^{II} + O_2 \quad (11)$$

This process cannot, however, be an important route for $OH^{\cdot}$ production from alloxan/GSH since although the formation of this radical was strongly inhibited by catalase, reflecting elimination of Reaction 10, SOD (which would inhibit Reaction 11) had little effect. Furthermore, iron–DPTA, which is a poor catalyst of the Haber–Weiss reaction [20] was an effective promoter of $OH^{\cdot}$ formation from alloxan and GSH. It is likely, therefore, that the catalytic cycle for $OH^{\cdot}$ formation in the present instance is maintained by reduction of Fe^{III} by dialuric acid [4]:

$$Fe^{III} + AH_2 \rightarrow Fe^{II} + AH^{\cdot} + H^+ \quad (12)$$

In the presence of iron, therefore, Reaction 12, which is fast [4], would replace the slow Reaction 4 as the initiation step of autoxidation; subsequent reactions of $AH^{\cdot}$ would be the same as in the uncatalyzed reaction.

The present investigation has shown that GSH and alloxan or dialuric acid can interact in a cyclic process which leads to GSH oxidation and free-radical production. If such reactions were to occur intracellularly, deleterious effects would be expected. However, in the physiological situation, GSH is accompanied by enzymes which moderate the cyclic reaction and these, acting in concert with the thiol, may constitute a powerful protective mechanism. In particular, SOD, in conjunction with GSH, maintained dialuric acid in the reduced form and prevented redox cycling through reduction of $AH^{\cdot}$ and inhibition of radical chain reactions. SOD, however, was unable to prevent $OH^{\cdot}$ formation from alloxan, GSH and a catalytic metal. In this case, catalase was a potent inhibitor and glutathione peroxidase, the other tissue enzyme capable of H_2O_2 destruction, would likewise be expected to limit $OH^{\cdot}$ formation. These antioxidant defences should therefore control the production of free-radical species from dialuric acid and prevent their interaction with other tissue components.

Such considerations imply that tissue damage would occur only when this defensive system is overwhelmed. This may be the case for divicine and isouramil [6], which cause haemolysis only in individuals whose erythrocytes are deficient in glucose-6-phosphate dehydrogenase; such cells are unable to maintain GSH levels under oxidative stress. Similarly, the pancreatic β-cells contain comparatively low levels of SOD, catalase and glutathione peroxidase [21], which, in conjunction with their avidity for the toxin, may explain the unusual susceptibility of this tissue to alloxan [3]. The ability

of thiols in combination with SOD to prevent redox cycling may also explain why pretreatment with GSH or cysteine can protect against alloxan diabetes in rats [22].

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