Alloxan-induced luminol luminescence as a tool for investigating mechanisms of radical-mediated diabetogenicity

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Chemiluminescence of luminol in a cell-free system was used to investigate the mechanism of alloxan-dependent free-radical generation. In the presence of alloxan and reduced glutathione (GSH), luminescence was greatly stimulated by FeSO_4 . Replacing GSH by oxidized glutathione or NAD(P)(H), or replacing FeSO_4 by CuSO_4 , ZnSO_4 or FeCl_3 , did not yield chemiluminescence. The chemiluminescence of a mixture of alloxan, GSH, FeSO_4 and luminol was inhibited by catalase, superoxide dismutase, scavengers of hydroxyl radicals (sodium benzoate, n-butanol, D-mannitol, dimethyl sulphoxide) or metal-ion chelators (EDTA, diethylenetriaminepenta-acetic acid, diethyldithiocarbamate, desferroxamine), D-glucose, L-glucose, D-mannose, D-fructose, 3-O-methyl-D-glucose, NAD^+ , NADH, NADP^+ or NADPH, but not by urea or enzymically inactive superoxide dismutase. The results support the hypothesis that the diabetogenic action of alloxan is mediated by hydroxyl radicals generated in an iron-catalysed reaction. Protection against alloxan *in vivo* depends both on the chemical reactivity of protector with radicals or radical-generating systems and on the stereospecific requirement of some strategic site in the B-cell.

By electron-spin-resonance studies Lagercrantz & Yhland (1963) demonstrated that the diabetogenic drug, alloxan, generates free radicals when mixed with glutathione, cysteine or ascorbic acid. On the basis of animal experiments, Heikkilä and co-workers (Heikkilä *et al.*, 1974, 1976; Heikkilä, 1977) proposed that hydroxyl radicals mediate the diabetogenic action of the drug. The following sequence of reactions may generate hydroxyl radicals ($\text{OH}^\bullet$): reduction of alloxan to dialuric acid, and the formation of superoxide radicals ($\text{O}_2^{\bullet-}$) by autoxidation back to alloxan (Deamer *et al.*, 1971; Cohen & Heikkilä, 1974); formation of H_2O_2 from $\text{O}_2^{\bullet-}$ and H^+ spontaneously or catalysed by superoxide dismutase (McCord & Fridovich, 1969); and formation of $\text{OH}^\bullet$ from $\text{O}_2^{\bullet-}$ and H_2O_2 according to the Haber–Weiss reaction as catalysed by iron (Halliwell, 1978*a,b*; McCord & Day, 1978) or perhaps other transition-metals (Van Hemmen & Meuling, 1977).

Studies *in vitro* with isolated islets or islet cells show that superoxide dismutase, catalase, scavengers of hydroxyl radicals and the metal-ion chelator DETAPAC protect against the cytotoxic

action of alloxan (Grankvist *et al.*, 1979*a,b*; Fischer & Hamburger, 1980). Urea reacts poorly with hydroxyl radicals (Anbar & Neta, 1967; Dorfman & Adams, 1973), and does not protect isolated islets or islet cells against alloxan (Grankvist *et al.*, 1979*a*). DETAPAC and desferroxamine are efficient inhibitors of hydroxyl-radical production by iron-catalysed reactions (Buettner *et al.*, 1978; Halliwell, 1978*a,b*; Gutteridge *et al.*, 1979), whereas EDTA and diethyldithiocarbamate stimulate some radical-yielding processes and inhibit others (cf. Halliwell, 1978*a*; Buettner *et al.*, 1978; Gutteridge *et al.*, 1979; Winterbourn, 1979; Goldstein *et al.*, 1979; Deneke & Fanburg, 1980). Also, some D-hexoses (but not L-hexoses), methylxanthines and pyridine nucleotides protect against the toxic action of alloxan on islet cells (Lacy *et al.*, 1975; Cooperstein & Watkins, 1977; Idahl *et al.*, 1977; Grankvist *et al.*, 1979*a*).

In the present study, luminol chemiluminescence was used to further elucidate the production of radicals by alloxan. The chemiluminescence of luminol is dependent on the relaxation to its ground state of a 3-aminophthalate di-anion excited by free radicals (Hodgson & Fridovich, 1973; O'Brien & Hulett, 1980). Basal conditions for optimum alloxan-induced luminescence were established and then the

effects of agents previously shown to protect against alloxan or to inhibit radical-generating systems were studied.

Materials and methods

Chemiluminescence measurements were performed in a liquid-scintillation spectrophotometer. Maximum signal-to-noise ratios were determined and measurements were performed in the ^3H channel with the coincidence function turned off (Webb *et al.*, 1974; Hodgson & Fridovich, 1975). The basal buffer (pH 7.4) had the same composition as Krebs-Ringer bicarbonate (DeLuca & Cohen, 1964), except that the bicarbonate was replaced by 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]. To avoid metal-ion contamination, the water was twice distilled and then de-ionized with chelating resin Chelex 100 (Bio-Rad Laboratories).

All measurements were performed at ambient room temperature (about 20°C). Polyethylene vials containing 5 ml of the buffer solution were kept in the dark overnight; vials that had not been so equilibrated showed high and variable background luminescence. All experiments were performed in the dark with the room lights turned off. Only the faint lights on the spectrophotometer display provided illumination. Portions of concentrated stock solutions were added to the vials to give the final concentrations listed in the text, Tables and Fig. 1. Compounds tested for inhibitory effects were dissolved in basal buffer and were added immediately before alloxan. Vials containing buffer only yielded background values of about 2000 counts/30 s. All results have been corrected for background.

When alloxan was added, a slight decrease in pH (at the most, down to 7.35) occurred. Chemiluminescence was recorded for 30 s. This was long enough to allow manual pipetting with only negligible variations in timing.

Alloxan monohydrate was from United States Biochemical Corp., Cleveland, OH, U.S.A. Bovine blood CuZn-superoxide dismutase was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; the enzyme activity was 2900 units/mg of protein as assayed by the method of McCord & Fridovich (1969). For control experiments, inactivated superoxide dismutase (provided by S. Marklund) was prepared by incubating the enzyme with H_2O_2 as previously described (Grankvist *et al.*, 1979a). Beef liver catalase containing 36000 units/mg of protein and 0.1% thymol was from Sigma Chemical Co.; 1 unit decomposed 1 μmol of H_2O_2 at 25°C, while the H_2O_2 concentration fell from 10.3 to 9.2 $\mu\text{mol}/\text{ml}$ of reaction mixture. Luminol, 3-O-methyl-D-glucose, D-glucose, L-glucose, L-fructose, theophylline, urea, DETAPAC, EDTA and diethyldithiocarbamate

were from Sigma Chemical Co. Pyridine nucleotides, glutathione (oxidized and reduced forms) and Hepes were from Boehringer-Mannheim G.m.b.H., Mannheim, Germany. Desferrioxamine B-methanesulphonate was from Ciba-Geigy, Basle, Switzerland. D-Mannose was from BDH Chemicals, Poole, Dorset, U.K. Chelex 100 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Sodium benzoate, n-butanol and D-mannitol were from Difco Laboratories, Detroit, MI, U.S.A. Dimethyl sulphoxide was from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. Other reagents were of analytical grade.

Results

Basal test conditions

A basal mixture of 100 μM -alloxan, 100 μM -GSH, 10 μM - FeSO_4 and 1 μM -luminol was highly luminescent. To characterize the system, the concentrations of the four reagents were systematically varied one at a time (results not shown). Chemiluminescence increased with the FeSO_4 concentration up to 10 μM , with luminol up to 5 μM , with GSH to 200 μM and with alloxan up to 100 μM . Higher concentrations of FeSO_4 , GSH or alloxan produced less luminescence. Light production from the basal system was approximately linear with time for at least 30 s (Fig. 1). In the presence of more than

Fig. 1. Alloxan-induced chemiluminescence in the presence of various concentrations of GSH

The chemiluminescence was recorded during the 30-s measuring period after mixing 100 μM -alloxan, 10 μM - FeSO_4 and 1 μM -luminol with various concentrations of GSH as indicated. Each curve is based on six separate experiments.

100 μ M-GSH, the time course was markedly non-linear.

As shown in Table 1, mixing FeSO_4 with luminol alone yielded light, as in other studies (Seitz & Hercules, 1972). The alloxan/GSH/ FeSO_4 mixture also exhibited a minor luminescence. The addition of GSH to the FeSO_4 /luminol mixture decreased the luminescence markedly. In contrast with these rather low intensities of luminescence, the addition of alloxan to the GSH/ FeSO_4 /luminol mixture yielded an intense light production. The combination of 100 μ M-alloxan, 100 μ M-GSH, 10 μ M- FeSO_4 and 1 μ M-luminol was therefore used in the following studies.

Effects of GSSG, NAD(P)⁺, NAD(P)H and various metal ions

To test whether GSSG, NAD(P)⁺ or NAD(P)H in combination with alloxan and luminol produced light in the presence of FeSO_4 , CuSO_4 or ZnSO_4 , 100 μ M-alloxan and 1 μ M-luminol was mixed with 10 μ M of a metal ion and 100 μ M of GSH, GSSG or a nucleotide. Three different observations were made on each mixture. With GSSG instead of GSH, no more light ($47\,291 \pm 2050$ counts/30 s; mean $\pm$ S.E.M.) was observed than with alloxan/ FeSO_4 /luminol alone ($45\,772 \pm 829$ counts/30 s). The minor light production from alloxan/ FeSO_4 /luminol was markedly inhibited by NADH

(587 ± 128) or NADPH (509 ± 43 counts/30 s) and to a lesser extent by NAD⁺ ($30\,386 \pm 1603$ counts/30 s) or NADP⁺ ($26\,471 \pm 549$ counts/30 s). Addition of FeCl_3 to alloxan/GSH/luminol did not affect luminescence, whereas CuSO_4 or ZnSO_4 was inhibitory (results not shown).

Effects of superoxide dismutase, catalase and hydroxyl-radical scavengers

Chemiluminescence was virtually abolished when superoxide dismutase or catalase was included in the basal luminescence system containing alloxan, GSH, FeSO_4 and luminol (Table 2). In contrast, inactivated superoxide dismutase did not decrease luminescence. D-Mannitol (20 mM), 20 mM-sodium benzoate or 53 mM-n-butanol markedly inhibited the alloxan-induced luminescence, whereas 28 mM-dimethyl sulphoxide had but a minor effect. Urea had no effect at all on the luminescence.

Effects of metal-ion chelators, hexoses and pyridine nucleotides

DETAPAC (20 μ M), 100 μ M-diethyldithiocarbamate, 20 μ M-EDTA or 20 μ M-desferrioxamine strongly inhibited luminescence. D-Glucose, L-glucose, D-mannose, D-fructose and 3-O-methyl-D-glucose, each at 20 mM, reduced the alloxan-induced luminescence (Table 2). 3-O-Methyl-D-glucose was the least effective sugar, with 57% inhibition, whereas the L- and D-forms of glucose, D-fructose and D-mannose inhibited by 73–83%. Both the reduced, and to a lesser extent, the oxidized forms of pyridine nucleotides inhibited the chemiluminescence.

Effects of metal ions and theophylline

CuSO_4 or ZnSO_4 (10 μ M) inhibited alloxan-induced luminescence, whereas 1 mM-theophylline had no effect (Table 2). An FeSO_4 solution, which had been kept at pH 10.0 overnight to convert Fe^{2+} into Fe^{3+} , did not induce luminescence when mixed with alloxan/GSH/luminol (results not shown).

Experiments in phosphate-free buffer

Because phosphate binds iron, the effect on luminescence of a buffer containing Hepes only (20 mM) was studied. In the presence of alloxan/GSH/luminol, FeSO_4 but not FeCl_3 caused luminescence to the same extent as when the salt-containing buffer was used (results not shown).

Discussion

In the presence of molecular O_2 and a reductant, alloxan and dialuric acid (the reduced form of alloxan) constitute a reduction–oxidation cycle that produces H_2O_2 (Deamer *et al.*, 1971) and free radicals (Lagercrantz & Yhland, 1963; Cohen & Heikkila, 1974). The extremely reactive hydroxyl

Table 1. Chemiluminescence of alloxan, luminol, FeSO_4 and GSH alone and in various combinations

Chemiluminescence of various agents added to the salt-balanced basal buffer was measured. Alloxan (100 μ M), 100 μ M-GSH, 10 μ M- FeSO_4 and 1 μ M-luminol were added alone or in combination as indicated. The background luminescence of buffer alone (2107 counts/30 s) has been corrected for. Results are means $\pm$ S.E.M. for four different observations.

Test agent	Luminescence (counts/30 s)
Alloxan	205 ± 31
Luminol	212 ± 46
FeSO_4	287 ± 55
GSH	21 ± 24
Luminol + alloxan	157 ± 69
Luminol + GSH	55 ± 38
Luminol + FeSO_4	$22\,991 \pm 1083$
Alloxan + FeSO_4	321 ± 81
Alloxan + GSH	1290 ± 205
FeSO_4 + GSH	545 ± 35
Luminol + alloxan + FeSO_4	$45\,772 \pm 829$
Luminol + alloxan + GSH	1757 ± 214
Luminol + GSH + FeSO_4	$11\,020 \pm 259$
Alloxan + GSH + FeSO_4	4866 ± 184
Luminol + alloxan + GSH + FeSO_4	$348\,677 \pm 9880$

Table 2. *Inhibition of alloxan-induced chemiluminescence by various agents*

Chemiluminescence of the basal mixture (100 μ M-alloxan, 100 μ M-GSH, 10 μ M-FeSO₄ and 1 μ M-luminol) was measured in the absence or presence of further additives as indicated. Results are means $\pm$ S.E.M. for four separate observations. Abbreviations used: DMSO, dimethyl sulphoxide; DDTC, diethyldithiocarbamate.

Additions to basal mixture	Luminescence (counts/30s)	Inhibition (%)
None	348 677 $\pm$ 9880	—
Catalase (18 000 units/ml)	4998 $\pm$ 437	99
Superoxide dismutase (0.5 mg/ml, equivalent to 1450 units/ml)	1975 $\pm$ 95	99
Inactivated superoxide dismutase (0.5 mg/ml)	307 274 $\pm$ 17 841	12
DMSO (28 mM)	323 428 $\pm$ 8459	7
D-Mannitol (20 mM)	31 642 $\pm$ 3024	91
Sodium benzoate (20 mM)	248 927 $\pm$ 9058	29
n-Butanol (53 mM)	227 992 $\pm$ 9631	35
Urea (20 mM)	345 171 $\pm$ 11 182	1
DETAPAC (20 μ M)	2833 $\pm$ 73	99
DDTC (100 μ M)	1841 $\pm$ 249	99
EDTA (20 μ M)	5135 $\pm$ 237	99
Desferroxamine (20 μ M)	2547 $\pm$ 108	99
D-Glucose (20 mM)	82 179 $\pm$ 604	77
L-Glucose (20 mM)	59 779 $\pm$ 4892	83
D-Mannose (20 mM)	67 963 $\pm$ 4992	81
D-Fructose (20 mM)	57 980 $\pm$ 4862	83
3-O-Methyl-D-glucose (20 mM)	150 418 $\pm$ 7990	57
GSSG (100 μ M)	235 222 $\pm$ 9380	33
NAD ⁺ (100 μ M)	250 709 $\pm$ 5923	28
NADH (100 μ M)	50 057 $\pm$ 761	86
NADP ⁺ (100 μ M)	232 464 $\pm$ 12 703	33
NADPH (100 μ M)	36 116 $\pm$ 2119	90
FeCl ₃ (10 μ M)	284 578 $\pm$ 9573	18
CuSO ₄ (10 μ M)	89 838 $\pm$ 3688	74
ZnSO ₄ (10 μ M)	35 313 $\pm$ 10 524	61
Theophylline (1 mM)	366 918 $\pm$ 11 634	0

radical (Anbar & Neta, 1967; Dorfman & Adams, 1973) may be formed by reduction of H₂O₂ with the superoxide radical (O₂^{-•}) acting as reductant (Haber & Weiss, 1934):

At physiological pH (7.4), the Haber–Weiss reaction requires catalysis by transitional metals such as iron (Cohen, 1977; Halliwell, 1978b; Czapski & Ilan, 1978; Koppenol *et al.*, 1978; Halliwell *et al.*, 1980):

Protection experiments *in vivo* and *in vitro* suggest that the hydroxyl radical is the damaging agent in alloxan-induced diabetes (Heikkilä *et al.*, 1974; Grankvist *et al.*, 1979a; Fischer & Hamburger, 1980).

The present results give further support for the free-radical hypothesis of alloxan cytotoxicity, and throw new light on three main points: (a) the relative

specificity of target groups in the initial production of dialuric acid; (b) the obligatory requirement for iron; (c) the relative importance of B-cell-dependent and B-cell-independent reactions in the protection afforded by various agents.

The present findings support the hypothesis that alloxan acts via thiol groups (Lazarow, 1949; Cooperstein *et al.*, 1964; Watkins *et al.*, 1979) because GSH, but not GSSG or reduced or oxidized pyridine nucleotides, reacted with alloxan to produce chemiluminescence. Moreover, reduced thio-redoxin, a reductant known to react rapidly with alloxan (Holmgren & Lyckeberg, 1980) and to be present in islet cells (Täljedal, 1981), caused an intense light production in the present assay system (K. Grankvist, A. Holmgren, M. Luthman & I.-B. Täljedal, unpublished work; Täljedal, 1981).

The presence of Fe²⁺ was obligatory for a marked production of light. This finding is compatible with the fact that the iron chelator DETAPAC prevents the toxic action of alloxan in isolated islets *in vitro* (Grankvist *et al.*, 1979b; Fischer & Hamburger, 1980). According to the Haber–Weiss formula, one

would expect Fe^{3+} also to be catalytic. The failure of FeCl_3 to catalyse the chemiluminescence may reflect a rapid autoxidation of Fe^{2+} , which is known to be particularly fast in the presence of chelators (Aisen, 1977). Hydroxyl radicals may be formed by a non-cycling oxidation of Fe^{2+} by H_2O_2 in the present system. However, the marked effect of superoxide dismutase clearly suggests a role for superoxide radicals in the system. This finding favours the Haber-Weiss reaction as a source of hydroxyl radicals.

The inhibitory effect of CuSO_4 on chemiluminescence could be due to Cu^{2+} -induced oxidation of GSH (Österberg *et al.*, 1979), $\text{O}_2^{\cdot-}$ (Rabani *et al.*, 1973) or Fe^{2+} (Kurimura & Murakami, 1969). Zn^{2+} binds to thiol groups (Willson, 1977; Rabenstein & Isab, 1980; Rabenstein *et al.*, 1979), and thus could prevent the reduction of alloxan.

The alloxan-induced luminescence was markedly inhibited by pyridine nucleotides, which also protect against alloxan in B-cells (Cooperstein & Watkins, 1977; Grankvist *et al.*, 1979a). Part of this protection could be due to oxidation of the reduced nucleotides by H_2O_2 and $\text{O}_2^{\cdot-}$ (Baccanari, 1978) and scavenging of $\text{OH}^{\cdot}$ (Anbar & Neta, 1967; Dorfman & Adams, 1973).

In the present study, both D- and L-glucose inhibited luminescence, whereas only D-glucose inhibits the toxic action of alloxan on B-cells. This indicates that the steric specificity of the islet B-cells plays a role in the protection of the cells against alloxan. The route for alloxan protection into the B-cells may be the stereospecific hexose transport (Hellman *et al.*, 1971; Scheynius & Täljedal, 1971; Idahl *et al.*, 1977). The idea that direct glucose receptors for the triggering of insulin secretion binds both glucose and alloxan has also been forwarded (Weaver *et al.*, 1978). That mechanisms other than a direct reaction with radicals are important for protection is also suggested by the failure of theophylline to inhibit luminescence. Theophylline is a strong protector against alloxan in B-cells (Lacy *et al.*, 1975; Grankvist *et al.*, 1979a). This discrepancy may be resolved by the fact that theophylline is an effective substrate for the hexose transport system (McDaniel *et al.*, 1977).

A puzzling observation was the minor effect of dimethyl sulphoxide on luminescence, as the drug is a potent hydroxyl-radical scavenger (Raleigh & Kremers, 1981) and protects against alloxan cytotoxicity (Grankvist *et al.*, 1979a). The reason for this discrepancy remained to be clarified. Perhaps the product of dimethyl sulphoxide and $\text{OH}^{\cdot}$ is in itself chemiluminescent.

Previous studies have disclosed several properties of the B-cells, which, when taken together, may favour the accumulation of free radicals in these cells. Thus B-cells appear to have a high con-

centration of reduced thiol groups (Havu, 1969), to contain thioredoxin and thioredoxin reductase (A. Holmgren, M. Luthman & I.-B. Täljedal, unpublished work; Täljedal, 1981), to possess only moderate endogenous activities of the enzymes superoxide dismutase, catalase and glutathione peroxidase (Grankvist *et al.*, 1981) and to possess sufficient amounts of metal ions as catalysts at the cell surface (Grankvist *et al.*, 1979b). These properties, together with the highly efficient and stereospecific hexose transport system, perhaps could explain why B-cells are particularly vulnerable to alloxan.

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