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 FeSO₄. Replacing GSH by oxidized glutathione or NAD(P)(H), or replacing FeSO₄ by CuSO₄, ZnSO₄ or FeCl₃, did not yield chemiluminescence. The chemiluminescence of a mixture of alloxan, GSH, FeSO₄ 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, desferoxamine), 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, Heikkila and co-workers (Heikkila et al., 1974, 1976; Heikkila, 1977) proposed that hydroxyl radicals mediate the diabetogenic action of the drug. The following sequence of reactions may generate hydroxyl radicals (OH·): reduction of alloxan to dialuric acid, and the formation of superoxide radicals (O₂⁻·) by autoxidation back to alloxan (Deamer et al., 1971; Cohen & Heikkila, 1974); formation of H₂O₂ from O₂⁻· and H⁺ spontaneously or catalysed by superoxide dismutase (McCord & Fridovich, 1969); and formation of OH· from O₂⁻· and H₂O₂ according to the Haber–Weiss reaction as catalysed by iron (Halliwell, 1978a,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., 1979a,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., 1979a). DETAPAC and desferoxamine are efficient inhibitors of hydroxyl-radical production by iron-catalysed reactions (Buettner et al., 1978; Halliwell, 1978a,b; Gutteridge et al., 1979), whereas EDTA and diethyldithiocarbamate stimulate some radical-yielding processes and inhibit others (cf. Halliwell, 1978a; 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., 1979a).

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 \(^3\)H 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 owing; 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/30s. 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.; I unit decomposed 1 \(\mu\)mol of \(H_2O_2\) at 25°C, while the \(H_2O_2\) concentration fell from 10.3 to 9.2 \(\mu\)mol/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. Desferroxamine B-methansulphonate was from Ciba-Geigy, Basle, Switzerland. D-Mannose 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 \(\mu\)M-alloxan, 100 \(\mu\)M-GSH, 10 \(\mu\)M-FeSO\(_4\) and 1 \(\mu\)M-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 \(\mu\)M, with luminol up to 5 \(\mu\)M, with GSH to 200 \(\mu\)M and with alloxan up to 100 \(\mu\)M. 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](image-url)
100 μM-GSH, the time course was markedly non-linear.

As shown in Table 1, mixing FeSO₄ with luminol alone yielded light, as in other studies (Seitz & Hercules, 1972). The alloxan/GSH/FeSO₄ mixture also exhibited a minor luminescence. The addition of GSH to the FeSO₄/luminol mixture decreased the luminescence markedly. In contrast with these rather low intensities of luminescence, the addition of alloxan to the GSH/FeSO₄/luminol mixture yielded an intense light production. The combination of 100 μM-alloxan, 100 μM-GSH, 10 μM-FeSO₄ and 1 μM-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 FeSO₄, CuSO₄ or ZnSO₄, 100 μM-allocyan and 1 μM-luminol was mixed with 10 μM of a metal ion and 100 μM of GSH, GSSG or a nucleotide. Three different observations were made on each mixture. With GSSG instead of GSH, no more light (47291 ± 2050 counts/30 s; mean ± S.E.M.) was observed than with alloxan/FeSO₄/luminol alone (45772 ± 829 counts/30 s). The minor light production from alloxan/FeSO₄/luminol was markedly inhibited by NADH + (587 ± 128) or NADPH (509 ± 43 counts/30 s) and to a lesser extent by NAD+ (30386 ± 1603 counts/30 s) or NADP+ (26471 ± 549 counts/30 s). Addition of FeCl₃ to alloxan/GSH/luminol did not affect luminescence, whereas CuSO₄ or ZnSO₄ 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, FeSO₄ 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**

DETPAC (20 μM), 100 μM-diethylthiocarbamate, 20 μM-EDTA or 20 μM-desferroxamine 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**

CuSO₄ or ZnSO₄ (10 μM) inhibited alloxan-induced luminescence, whereas 1 μM-theophylline had no effect (Table 2). An FeSO₄ solution, which had been kept at pH 10.0 overnight to convert Fe²⁺ into Fe³⁺, 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, FeSO₄ but not FeCl₃ caused luminescence to the same extent as when the salt-containing buffer was used (results not shown).

**Discussion**

In the presence of molecular O₂ and a reductant, alloxan and dialuric acid (the reduced form of alloxan) constitute a reduction–oxidation cycle that produces H₂O₂ (Deamer et al., 1971) and free radicals (Lagercrantz & Yhland, 1963; Cohen & Heikkila, 1974). The extremely reactive hydroxyl
Table 2. Inhibition of alloxan-induced chemiluminescence by various agents
Chemiluminescence of the basal mixture (100 μM-alloxan, 100 μM-GSH, 10 μM-FeSO₄ and 1 μM-luminol) was measured in the absence or presence of further additives as indicated. Results are means ± S.E.M. for four separate observations. Abbreviations used: DMSO, dimethyl sulfoxide; DDTC, diethyldithiocarbamate.

<table>
<thead>
<tr>
<th>Additions to basal mixture</th>
<th>Luminescence (counts/30s)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>348 677 ± 9880</td>
<td>—</td>
</tr>
<tr>
<td>Catalase (18 000 units/ml)</td>
<td>4998 ± 437</td>
<td>99</td>
</tr>
<tr>
<td>Superoxide dismutase (0.5 mg/ml, equivalent to 1450 units/ml)</td>
<td>1975 ± 95</td>
<td>99</td>
</tr>
<tr>
<td>Inactivated superoxide dismutase (0.5 mg/ml)</td>
<td>307 274 ± 17 841</td>
<td>12</td>
</tr>
<tr>
<td>DMSO (28 mM)</td>
<td>323 428 ± 84 59</td>
<td>7</td>
</tr>
<tr>
<td>D-Mannitol (20 mM)</td>
<td>31 642 ± 30 24</td>
<td>91</td>
</tr>
<tr>
<td>Sodium benzoate (20 mM)</td>
<td>248 927 ± 90 58</td>
<td>29</td>
</tr>
<tr>
<td>n-Butanol (53 mM)</td>
<td>227 992 ± 96 31</td>
<td>35</td>
</tr>
<tr>
<td>Urea (20 mM)</td>
<td>345 171 ± 11 82</td>
<td>1</td>
</tr>
<tr>
<td>DETAPAC (20 μM)</td>
<td>2833 ± 73</td>
<td>99</td>
</tr>
<tr>
<td>DDTC (100 μM)</td>
<td>1841 ± 249</td>
<td>99</td>
</tr>
<tr>
<td>EDTA (20 μM)</td>
<td>5135 ± 237</td>
<td>99</td>
</tr>
<tr>
<td>Desferroxamine (20 μM)</td>
<td>2547 ± 108</td>
<td>99</td>
</tr>
<tr>
<td>D-Glucose (20 mM)</td>
<td>82 179 ± 604</td>
<td>77</td>
</tr>
<tr>
<td>L-Glucose (20 mM)</td>
<td>59 779 ± 48 92</td>
<td>83</td>
</tr>
<tr>
<td>D-Mannose (20 mM)</td>
<td>67 963 ± 49 92</td>
<td>81</td>
</tr>
<tr>
<td>D-Fructose (20 mM)</td>
<td>57 980 ± 48 62</td>
<td>83</td>
</tr>
<tr>
<td>3-O-Methyl-d-glucose (20 mM)</td>
<td>150 418 ± 79 90</td>
<td>57</td>
</tr>
<tr>
<td>GSSG (100 μM)</td>
<td>235 222 ± 93 80</td>
<td>33</td>
</tr>
<tr>
<td>NAD⁺ (100 μM)</td>
<td>250 709 ± 59 23</td>
<td>28</td>
</tr>
<tr>
<td>NADH (100 μM)</td>
<td>500 57 ± 761</td>
<td>86</td>
</tr>
<tr>
<td>NADP⁺ (100 μM)</td>
<td>232 464 ± 12 703</td>
<td>33</td>
</tr>
<tr>
<td>NADPH (100 μM)</td>
<td>36 116 ± 21 19</td>
<td>90</td>
</tr>
<tr>
<td>FeCl₃ (10 μM)</td>
<td>284 578 ± 95 73</td>
<td>18</td>
</tr>
<tr>
<td>CuSO₄ (10 μM)</td>
<td>89 838 ± 36 88</td>
<td>74</td>
</tr>
<tr>
<td>ZnSO₄ (10 μM)</td>
<td>35 313 ± 10 524</td>
<td>61</td>
</tr>
<tr>
<td>Theophylline (1 mM)</td>
<td>366 918 ± 11 634</td>
<td>0</td>
</tr>
</tbody>
</table>

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):

\[
H₂O₂ + O₂⁻⁻ \rightarrow OH^- + OH^- + O₂
\]

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):

\[
Fe^{3+} + O₂⁻⁻ \rightarrow Fe^{2+} + O₂
\]

\[
Fe^{2+} + H₂O₂ \rightarrow Fe^{3+} + OH^- + OH^- + O₂
\]

Protection experiments in vivo and in vitro suggest that the hydroxyl radical is the damaging agent in alloxan-induced diabetes (Heikkila 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 thioredoxin, 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
Alloxan-induced luminescence would expect Fe$^{2+}$ 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$_2$O$_2$ 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), O$_2^-$ (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$_2$O$_2$ and O$_2^-$ (Baccanari, 1978) and scavenging of OH$^-$ (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 & Taljedal, 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 & Kremer, 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 OH$^-$ 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 concentration of reduced thiol groups (Havu, 1969), to contain thioredoxin and thioredoxin reductase (A. Holmgren, M. Luthman & I.-B. Taljedal, unpublished work; Taljedal, 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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