Oxidative metabolism and heat shock-enhanced chemiluminescence in *Dictyostelium discoideum*

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Summary

During early differentiation starving *Dictyostelium discoideum* amoebae produce a burst of light that is enhanced by heat shock at the beginning of development. We report here pharmacological, genetic and spectral evidence that the chemiluminescence results from lipid peroxidation reactions following oxygen reduction by leakage of electrons from ubiquinone in mitochondrial electron transport, and perhaps by peroxisomal oxidation of urate. Our results are consistent with the view that heat shock causes oxidative stress, which in turn induces heat shock proteins and production of reduced oxygen metabolites.

Key words: free radicals, heat shock, chemiluminescence, *Dictyostelium*, mitochondria, peroxisomes.

Introduction

Partially reduced forms of oxygen (O_2^-, H_2O_2, $OH^\cdot$) are cytotoxic and may contribute to the ageing process as well as to the pathology of age-related and autoimmune diseases and of tissue damage after oxidative stress (Pryor, 1986; McCord, 1987). Reduced oxygen metabolites participate in reaction cascades leading to light emission so that chemiluminescence is widely used to measure their production (Cadenas *et al.* 1984). We reported that *Dictyostelium discoideum* amoebae chemiluminesce during early differentiation and that light emission is enhanced by heat shock at the beginning of development (Fisher and Rosenberg, 1988). These results were consistent with suggestions that perturbed mitochondrial oxidative metabolism might generate intracellular stress signal(s) controlling gene expression in stressed cells.

Mitochondria are not the only site of oxidative metabolism and production of reduced oxygen metabolites. Except for the terminal cytochrome oxidase in respiratory electron transport, almost all oxidases only partially reduce oxygen, producing either O_2^- or H_2O_2 by one- or two-electron transfers to molecular oxygen (Pryor, 1986; Fridovich, 1976). As the first step in determining the pathways leading to light emission in *D. discoideum* we tested the effects of a variety of pharmacological agents on heat-shock-enhanced chemiluminescence. Two categories of compounds were tested. (1) Inhibitors or substrates of enzymes involved in oxidative metabolism. (2) Traps or producers of chemical species involved in oxygen free radical reactions.

The results suggest that in *D. discoideum* chemiluminescence results from oxygen reduction by leakage of electrons from ubiquinone in mitochondrial electron transport, and perhaps by peroxisomal oxidation of urate. This conclusion is supported further by genetic evidence showing that catalase deficiency associated with a mutation in the *catA* locus on linkage group II leads to enhanced chemiluminescence.

Materials and methods

Strains

The genotype and origin of each of the strains used in this work are described in Table 1. All genetically marked strains are descended from the wild-type strain NC4 and were constructed by the standard procedures of classical genetics in this organism (Loomis, 1987). Unless otherwise specified all results reported here were obtained using strain X22, which is catalase deficient (Madigan and Katz, 1989) and strongly chemiluminescent (Fisher and Rosenberg, 1988).

Catalase tests

Cells were scraped from the edge of a colony growing on a lawn of *Klebsiella aerogenes* on nutrient agar and suspended in 1.5 ml sterile Bonner's salts solution (0.6 g l^{-1} NaCl, 0.75 g l^{-1} KCl, 0.4 g l^{-1} $CaCl_2.2H_2O$). The amoebae were washed 3 times to remove bacteria by brief differential centrifugation in an Eppendorf centrifuge (total spin time 20 s). The presence of catalase was indicated by the formation of O_2 bubbles after addition of 5 μl of 27 % H_2O_2 to the final wet pellet.

Chemiluminescence experiments

Each experiment was performed on at least two separate occasions and unless otherwise noted results from single representative experiments are reported here. *D. discoideum* amoebae were harvested after growth on nutrient agar in association with *Klebsiella aerogenes* as previously (Fisher and Rosenberg, 1988). After being washed free of bacteria by differential centrifugation, $\sim 5\times10^7$ amoebae were spread onto the surface of a thin layer of water agar in each of several glass scintillation vials. The water agar was supplemented with appropriate concentrations of the chemical agent being tested. Vials were incubated at either 21.5 °C or 33 °C for 6–14 h as previously (Fisher and Rosenberg, 1988). The chemiluminescence results shown here pertain to vials incubated at 33 °C, but in all

Table 1. *Genotype and origin of strains used*

Strain	Parent	Linkage group I	II	III	IV	VI	VII	Reference
NC4								Raper (1935)
X22	DP4	*sprA1* *tsgE13*	*whiA1* *acrA1* *tsgD12* *catA*					Williams and Newell (1976)
HPF3	HU1628	*cycA1*	*acrA1823*	*bsgA5*	*whiC351* *phoD2317*	*manA2*	*tsgK21* *couA351*	This paper
HPF7	X22	*sprA1* *tsgE13*	*whiA1* *acrA1* *tsgD12* *catA*	*phoE2305*				This paper
HPF18	DPF13	*cycA1*	*whiA1* *tsgD12* *catA*	*bsgA5*		*manA2*		This paper
HPF19	DPF13	*cycA1*	*acrA1823*	*bsgA5*			*tsgK21* *couA351*	This paper
HPF25	DPF13	*cycA1*	*whiA1* *acrA1* *tsgD12* *catA*	*bsgA5*				This paper
HPF26	DPF13	*cycA1*	*acrA1823*	*bsgA5*			*tsgK21* *couA351*	This paper
HPF27	DPF13	*cycA1*	*acrA1823*	*bsgA5*		*manA2*		This paper
HPF30	DPF13	*cycA1*	*acrA1823*	*bsgA5*	*whiC351* *phoD2317*	*manA2*		This paper

Phenotypes associated with genotypic symbols: *spr*, round spores; *whi*, white (non-yellow) spores; *tsg*, temperature sensitive for growth at 27 °C; *acr*, resistant to acriflavin; *cat*, catalase deficient; *cyc*, resistant to cycloheximide; *bsg*, unable to grow on a *Bacillus subtilis* lawn; *man*, α-mannosidase deficient; *pho*, impaired slug phototaxis; *cou*, sensitive to coumarin. DPF13 is a diploid constructed by crossing HPF3 and HPF7.

cases, similar results were observed for vials incubated at 21.5 °C in which the chemiluminescence was not enhanced by heat shock. Chemiluminescence measurements were made every 30 min using a New Brunswick Lumitran ATP Photometer (Fisher and Rosenberg, 1988). Total light emission over the period of the experiment was obtained as the area under the curve of chemiluminescence (cts min^{-1} after background subtraction) *versus* time (min). It is expressed here as a percentage of the total light emission by amoebae in control vials containing water agar unsupplemented with the chemical agent being tested. The inhibition or enhancement of total light emission observed with the various chemical agents was reproducible in different experiments to within ±25 % of control values.

After each experiment all vials were incubated a further 48 h at 21.5 °C and scored for normal development (formation of fruiting bodies). Unless otherwise indicated, aggregates and fruiting bodies were absent after 48 h at 21.5 °C in all vials exposed to 33 °C during the period of the chemiluminescence experiment. This is a result of cell death at the heat shock temperature during the latter part of the experiment when chemiluminescence is declining (Fisher and Rosenberg, 1988, and unpublished data). Impaired development in control vials incubated throughout at 21.5 °C was taken as a presumptive indicator of cytotoxicity of particular chemical agents. Unless otherwise indicated, development at 21.5 °C was unaffected by the presence of each of the compounds used at the concentrations tested.

Results

Participation of hydroxyl radicals in chemiluminescence

Mannitol, glycerol and allopurinol intercept and react with hydroxyl radicals ($OH^{\cdot}$), making them unavailable for participation in light-yielding or other pathways (Moorhouse *et al.* 1987). Allopurinol also inhibits xanthine oxidase whose activity generates $O_2^{\cdot-}$ and H_2O_2 (Simpson *et al.* 1987). When present in the agar at concentrations greater than 100 μM, each of these $OH^{\cdot}$ traps inhibited chemiluminescence (Fig. 1A,B,C); 50 % inhibition occurred in the range from 0.1 mM to 2 mM and in the case of mannitol (Fig. 1A) and glycerol (Fig. 1B) almost total inhibition was found at 100 mM. These results suggest that hydroxyl radicals play a role in *D. discoideum* chemiluminescence as they do in other organisms. Unexpectedly, fruiting bodies were formed at normal densities in 33 °C vials containing 10 mM or 100 mM mannitol or glycerol. This result raises the possibility that cell death at 33 °C might be due to damage by heat shock-induced hydroxyl radicals. In other experiments, 10 mM DL-histidine, which also traps hydroxyl radicals, inhibited chemiluminescence by about 50 % (not shown). The organic acid anions benzoate and formate, which also trap hydroxyl radicals, failed to inhibit light emission at 10 mM (not shown), perhaps because of a failure to accumulate at sufficiently high concentrations in the cell.

Participation of hydrogen peroxide in chemiluminescence

Pharmacological evidence. To test the potential role of H_2O_2 in light-yielding pathways we tested the effects on chemiluminescence of H_2O_2, catalase, and the catalase inhibitors 3-amino-1,2,4-triazole and pyrazole (Jain, 1982). The catalase inhibitors were tested using the wild-type strain NC4 which, unlike X22, contains normal levels of catalase. Hydrogen peroxide at concentrations greater than about 1 mM induced premature light emission, while lower concentrations down to about 10 μM enhanced chemiluminescence at the normal time of development (Fig. 2). Catalase at concentrations $\geq 100\ \mu g\ ml^{-1}$ inhibited light production (Fig. 3), but the

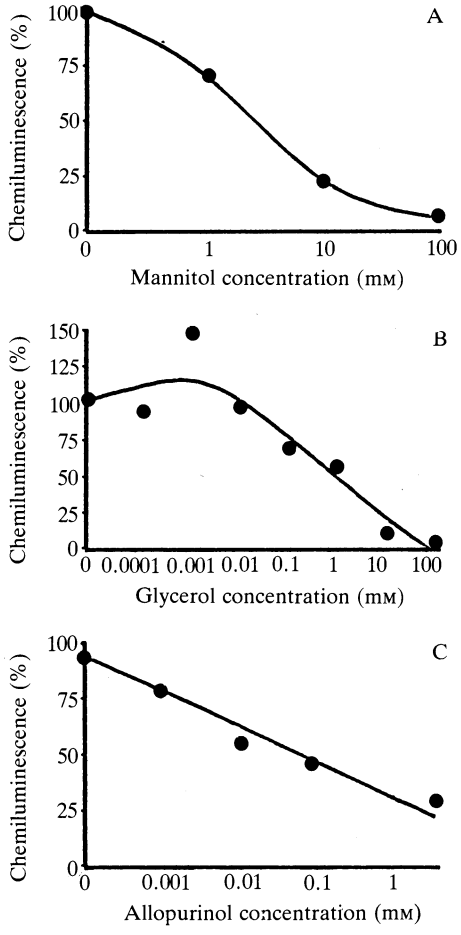

Fig. 1. Inhibition of chemiluminescence by the $OH^{\cdot}$ traps mannitol (A), glycerol (B) and allopurinol (C). Total chemiluminescence, expressed as a percentage of that found in control vials, was estimated as the area under the curve of chemiluminescence (cts min^{-1}) *versus* development time (cf. Fig. 2). Each vial contained ~5×10^7 starving amoebae of strain X22 on a thin layer of 1 mM Tris–agar (pH 7.5) at 33 °C. Subsequent fruiting body formation at 21 °C over 48 h was normal in vials containing 10 mM or 100 mM glycerol or mannitol. In other vials incubated at 33 °C subsequent development at 21 °C was impaired as expected. Post-aggregation development was normal in all control vials incubated throughout at 21 °C, indicating that even at 100 mM the $OH^{\cdot}$ traps used were not cytotoxic.

two catalase inhibitors had no effect (Fig. 4A,B). The effect of catalase in particular indicates a role for hydrogen peroxide in light production.

The negative results with the catalase inhibitors might be due to their inability to permeate the *D. discoideum* membrane, but an alternative explanation is suggested by experiments in which aminotriazole inhibited chemiluminescence in X22 (not shown). This suggests that in NC4 the expected enhancement of chemiluminescence by catalase inhibitors may be masked by a concurrent inhibition of chemiluminescence.

Genetic evidence. In many experiments X22 was more strongly chemiluminescent than many other strains, including NC4. This prompted us to measure chemiluminescence in selected segregants from a genetically well-marked diploid strain DPF13, made by crossing the haploid strains HPF3 and HPF7. HPF3 was derived by spontaneous loss of the unstable $CoCl_2$ resistance from the strain HU1628. HPF7 is a phototaxis mutant of X22 isolated after chemical mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by previously described methods (Fisher and Williams, 1982). HPF7, like its parent X22, was found to be strongly chemiluminescent, as were each

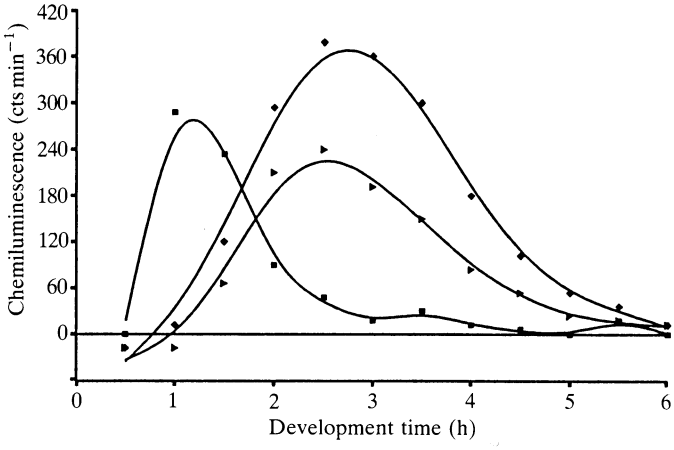

Fig. 2. Enhancement of chemiluminescence by H_2O_2. Chemiluminescence (cts min^{-1}) was measured for starving X22 amoebae at 33 °C on Tris–agar (pH 7.5) containing 0 (▶), 88 μM (◆) or 8.8 mM (■) H_2O_2. The nominal development time is the time in hours since the amoebae were washed free of bacteria by differential centrifugation. In this and other experiments, post-aggregation development was normal in control vials incubated at 21 °C that contained less than 1 mM H_2O_2 but was impaired in all vials exposed to 33 °C during the experiment. Some impairment of development (fewer than normal slugs after 24 h) was evident at 21 °C in the vial containing 8.8 mM H_2O_2. Other details as for Fig. 1. In other experiments 40 mM H_2O_2 and higher concentrations were found to be cytotoxic (development completely inhibited in 21 °C control vials) and to induce premature chemiluminescence.

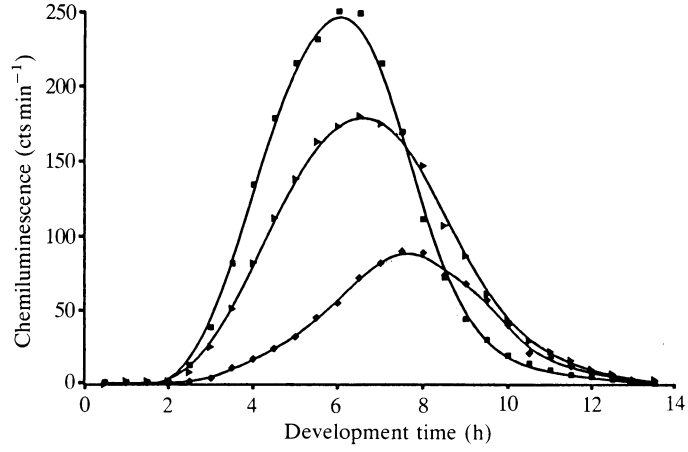

Fig. 3. Inhibition of chemiluminescence by catalase. Catalase was incorporated into the Tris–agar at 0 (■), 145 μg ml^{-1} (▶) or 1.45 mg ml^{-1} (◆). In another experiment using fewer cells and with lower chemiluminescence in the controls, light emission was totally abolished at the highest catalase concentration. If, instead of being incorporated into the agar, the enzyme was spread onto the surface with the amoebae, 1.45 μg catalase was sufficient to reduce chemiluminescence to 30% of control values. No evidence of cytotoxicity was found in control vials incubated throughout at 21 °C. Other details as for Fig. 1.

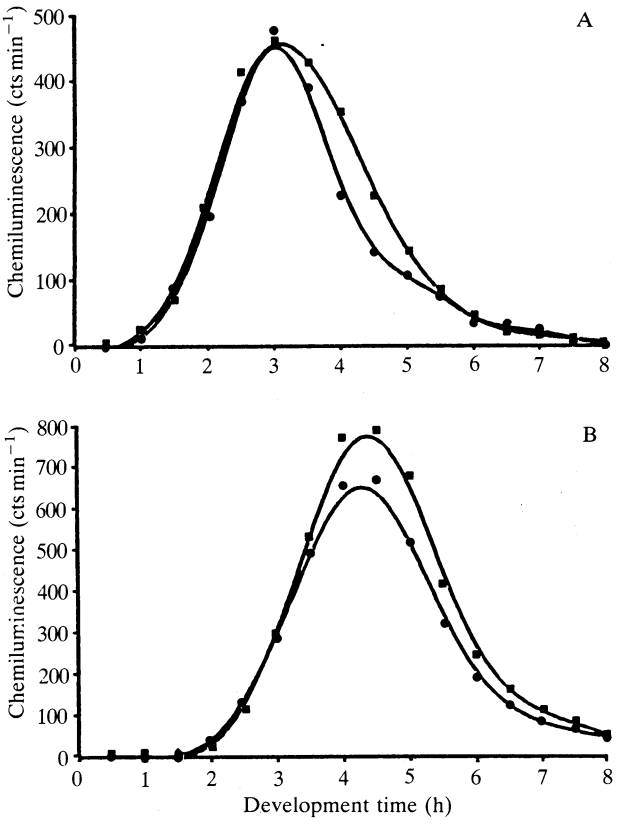

Fig. 4. Absence of effects on chemiluminescence in strain NC4 of two catalase inhibitors: (A) 10 mM pyrazole (●); and (B) 17 mM 3-amino-1,2,4-triazole (●). Control vials contained Tris–agar not supplemented with the inhibitor in each case (■). Higher concentrations were not tested and lower concentrations also had no effect. Other details as for Fig. 2.

of the segregants carrying the X22-derived linkage group II (Table 1, Table 2). Strong chemiluminescence was not consistently observed in HPF3 or in haploid segregants carrying the HPF3-derived linkage group II (Table 1, Table 2). The level of chemiluminescence was not correlated with any of the other marked linkage groups in these strains. These results suggested that the gene(s) responsible for strong chemiluminescence in X22 reside on linkage group II.

Madigan and Katz (1989) reported recently that X22 is catalase deficient because of a mutation in the *catA* locus on linkage group II. Catalase tests on our strains confirmed this and show that strong chemiluminescence is genetically linked to the catalase deficiency (Table 2). These results provide genetic evidence that H_2O_2 and catalase play a role in *Dictyostelium* chemiluminescence.

Superoxide and SOD participate in chemiluminescence

Diethyl carbamate is an inhibitor of the cytoplasmic Cu^{2+}/Zn^{2+} superoxide dismutase (SOD) of eukaryotes (Jain, 1982). When added to the agar at a concentration of 10 μM or 100 μM, it inhibited chemiluminescence (Fig. 5), whereas phenazine methosulphate, which participates in O_2^--yielding reactions (Nishikimi *et al.* 1972; Docampo and Moreno, 1984), enhanced light emission (Fig. 6). The results suggest that superoxide and cytoplasmic SOD are important in the light-yielding pathways in *Dictyostelium* and that the conversion of O_2^- to H_2O_2 is rate limiting for chemiluminescence. Consistent with this, we found that

Table 2. *Genetic linkage of strong chemiluminescence to catalase deficiency*

	Number of strains with indicated phenotype			
	Strong chemiluminescence at 21 °C		Strong chemiluminescence at 33 °C	
Phenotype	+	−	+	−
Cat^-	3	0	3	0
Cat^+	0	5	1	4

Cat^+, presence, or Cat^-, absence of catalase. Scoring of chemiluminescence was based on mean total light emission from two independent experiments for each strain. Strains included in this series of experiments and listed in the table were the parental strains HPF3, HPF7 and the segregants from DPF13 listed in Table 1. Mean chemiluminescence values fell into two groups, both for chemiluminescence at 21 °C and for heat-shock-enhanced chemiluminescence at 33 °C. At 21 °C total light emission for each of the strongly chemiluminescent strains was >450 counts/10^6 cells, and <400 counts/10^6 cells for each of the other strains. At 33 °C total light emission was >4200 counts/10^6 cells for the strongly chemiluminescent strains and <3200 counts/10^6 cells for the others. One catalase-containing strain HPF27 was scored as strongly chemiluminescent at 33 °C but not at 21 °C. This strain could be mis-scored at 33 °C as the extent of heat shock enhancement of chemiluminescence depends very sensitively on the temperature and timing with respect to development of the heat shock (Fisher and Rosenberg, 1988).

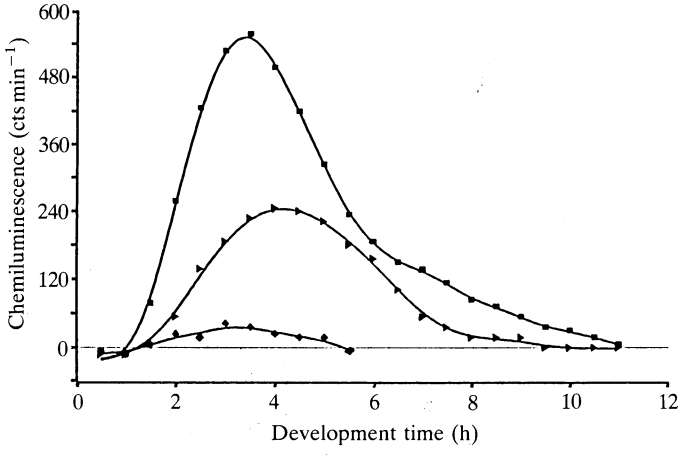

Fig. 5. Inhibition of chemiluminescence by the superoxide dismutase inhibitor diethyl carbamate. The concentration of diethyl carbamate in the Tris–agar was 0 (■), 10 μM (▶), or 100 μM (◆). Higher concentrations up to 100 mM were also inhibitory, but were cytotoxic, as indicated by impaired morphogenesis in control vials incubated throughout at 21 °C. Other details as for Fig. 2.

SOD caused a slight increase in the amount of light produced (Fig. 7). SOD may intercept and convert O_2^- that escapes the cell into H_2O_2, which can then participate in light-yielding reactions. Alternatively, SOD could enhance chemiluminescence by its known light-yielding reaction with H_2O_2.

Iron-catalysed reactions are important in chemiluminescence

Hydroxyl radicals, hydrogen peroxide and superoxide are interconverted spontaneously and in the presence of Fe^{2+}/Fe^{3+} by cascades of reactions known collectively as the Haber-Weiss reaction (Fridovich, 1976; Cadenas *et al.*

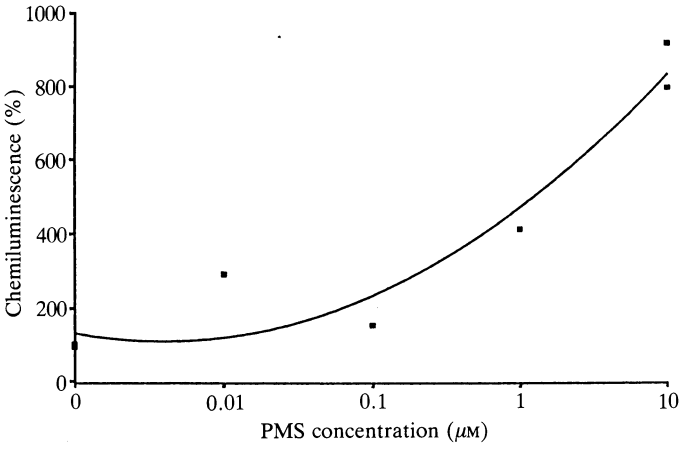

Fig. 6. Enhancement of chemiluminescence by phenazine methosulphate (PMS), which participates in reactions that generate O_2^-. Phenazine methosulphate was incorporated into the Tris–agar at the indicated concentrations. At higher concentrations (100 μM) cytoxicity at 21 °C was observed and chemiluminescence was inhibited. Other details as for Fig. 1.

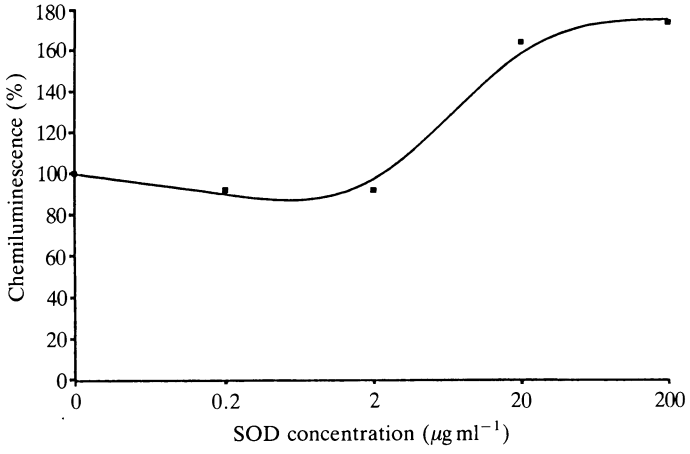

Fig. 7. Enhancement of chemiluminescence by superoxide dismutase. Bovine SOD was incorporated into the Tris–agar at the indicated concentrations. Chemiluminescence was estimated as total counts, expressed as a percentage of control values and the mean taken from 3 experiments. In other experiments 2 mg ml^{-1} SOD also enhanced chemiluminescence to a similar extent as shown here for 20 μg ml^{-1} and 200 μg ml^{-1}. No evidence of cytotoxicity was found. Other details as for Fig. 1.

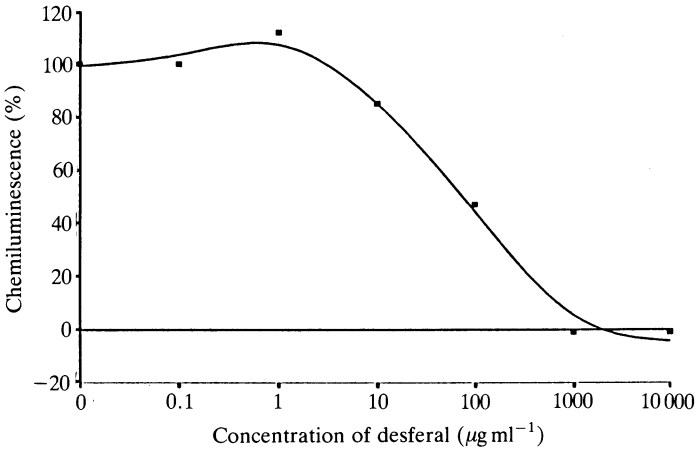

Fig. 8. Inhibition of chemiluminescence by the iron-chelating agent desferal. Desferal (desferrioxamine) was incorporated into the Tris–agar at the indicated concentrations. No evidence for cytotoxicity was found. Other details as for Fig. 1.

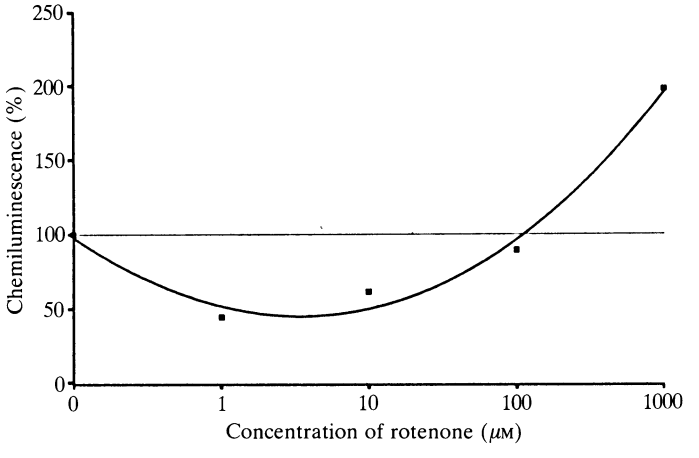

Fig. 9. Effect on chemiluminescence of the electron transport inhibitor rotenone. Rotenone was incorporated into the agar at the concentrations indicated. Total light emission over the course of the experiment was estimated, expressed as a percentage of the values in control vials and means taken of data from each of 4 experiments. The amount of chemiluminescence at the highest concentration used is inflated because of one experiment in which we observed very strong enhancement of chemiluminescence. At 100 μM and 1 mM development was inhibited in control vials not exposed to the heat-shock temperatures, indicating cytoxicity. No toxic effects on normal development were observed at the lower concentrations where chemiluminescence was always inhibited. Other details as for Fig. 1.

1984). Free iron is also necessary for the lipid peroxidation reactions that accompany chemiluminescence in mammalian tissues (Cadenas *et al.* 1984; Gutteridge *et al.* 1979). When we added the iron-chelating agent desferal to the agar chemiluminescence was inhibited (Fig. 8, 50% inhibition at <100 μg ml^{-1}). This result indicates that iron-catalysed reactions (Haber-Weiss or lipid peroxidation or both) play a role in the pathways leading to light emission.

Leaky mitochondrial electron transport and chemiluminescence

Mitochondrial electron transport in *D. discoideum* is similar to that in mammalian cells (Erikson and Ashworth, 1969). Ubiquinone is known to be capable of donating electrons directly to molecular oxygen to form superoxide (Loschen *et al.* 1974). To test for the possibility that leaky mitochondrial electron transport might be the source of superoxide involved in light emission, we added inhibitors of electron transport to the agar. Rotenone, which blocks reduction of ubiquinone by NADH dehydrogenase, inhibited chemiluminescence at concentrations of 1 μM and 10 μM (Fig. 9). This would be expected if chemiluminescence results from direct donation of electrons by the reduced form of ubiquinone to molecular O_2 to form O_2^-. At higher concentrations, which were clearly

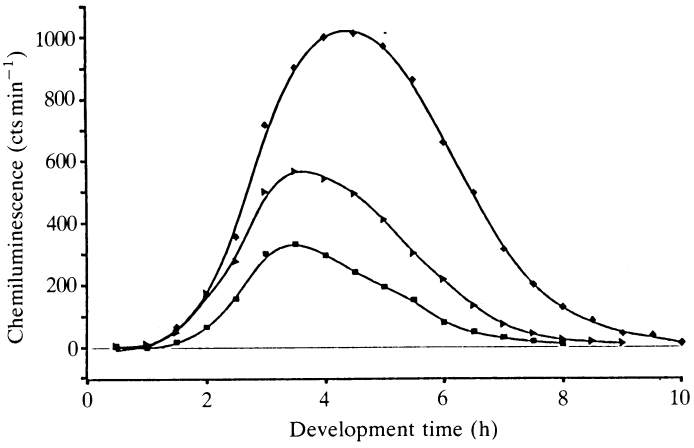

Fig. 10. Enhancement of chemiluminescence by antimycin A, an electron transport inhibitor. Antimycin A was incorporated into the agar at 0 μM (■), 2 μM (▶) and 20 μM (◆). Enhancement similar to that occurring at 2 μM was also observed at 200 μM. No evidence of cytoxicity at 21 °C was found at any of the concentrations used. Other details as for Fig. 2.

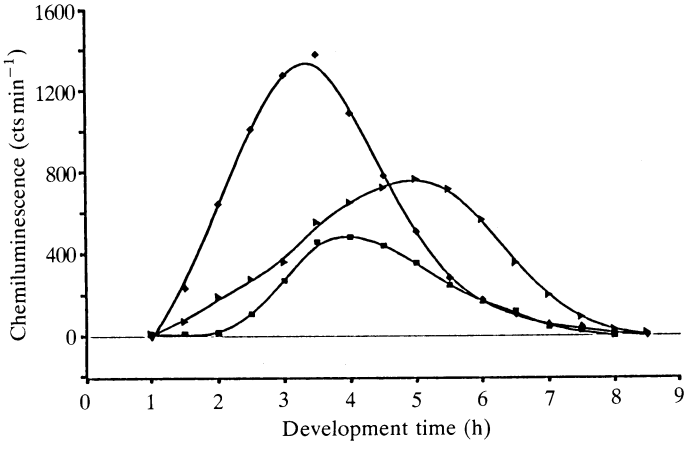

Fig. 11. Enhancement of chemiluminescence by the electron transport inhibitor dibromothymoquinone. The inhibitor was incorporated into the agar at a concentration of 0 (■), 150 μM (▶), or 1.5 mM (◆). At these concentrations, development in control vials not exposed to the heat shock temperatures was normal. At higher concentrations (15 mM and 150 mM) a brief premature burst of chemiluminescence was observed, and the lack of normal development in 21 °C control vials indicated cytoxicity. Other details as for Fig. 2.

cytotoxic, this inhibition was offset by an apparent enhancement (Fig. 9) that could have arisen because of induction of chemiluminescence by oxidative stress. More light was produced in the presence of 20 μM or 200 μM antimycin A, which blocks electron transport from ubiquinone to cytochrome *c* by ubiquinone-cytochrome *c* reductase (Fig. 10). Chemiluminescence was also enhanced by 150 μM or 1.5 mM dibromothymoquinone, which competes with ubiquinone for electrons but donates them to oxygen instead of passing them on to cytochrome *b* (Loschen and Azzi, 1974) (Fig. 11). Antimycin A and dibromothymoquinone could enhance chemiluminescence by causing both oxidative stress and increased diversion of

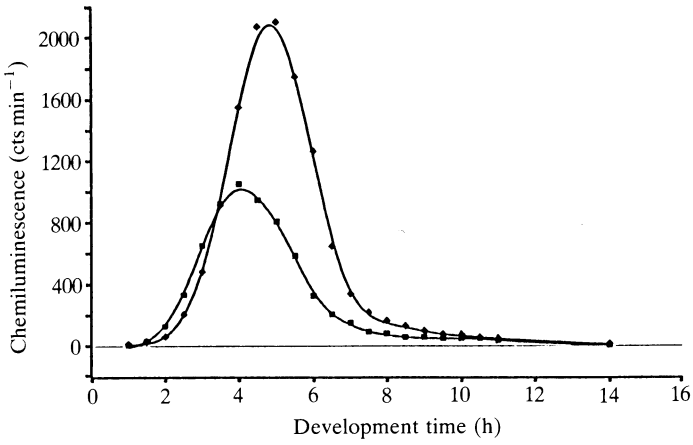

Fig. 12. Enhancement of chemiluminescence by urate. Urate at a concentration of 0 (■), or 1 mM (◆) was incorporated into the Tris–agar. Lower urate concentrations had no effect, and no cytotoxicity was observed in 21 °C control vials at any urate concentration. Other details as for Fig. 2.

electrons from ubiquinone to direct reduction of O_2 to O_2^-. The results with all three inhibitors of mitochondrial electron transport indicate that during chemiluminescence electrons leak to molecular O_2 from ubiquinone.

Peroxisomal urate oxidase may play a role in chemiluminescence

Like other eukaryotic cells, *D. discoideum* amoebae have peroxisomes containing catalase and at least one H_2O_2-generating oxidase. In the case of *D. discoideum* the only known peroxisomal oxidase is urate oxidase (Parish, 1975; Hayashi and Suga, 1978). We added the substrate urate, or the competitive urate oxidase inhibitors violurate and 2,6,8-trichloropurine (Muller and Moller, 1969), to the agar in the vials. Chemiluminescence was enhanced in the presence of 1 mM urate (Fig. 12) but inhibited by both urate oxidase inhibitors at a concentration of 10 mM (Fig. 13A,B). *Dictyostelium discoideum* urate oxidase is inhibited *in vitro* by 2,6,8-trichloropurine with a K_i of 1 μM (Parish, 1975). The high concentration required to inhibit chemiluminescence *in vivo* could result from poor permeation of the cell membrane by the inhibitor. The results with urate and the two inhibitors are consistent with a role for peroxisomal urate oxidase in the pathways leading to light emission in *Dictyostelium*. Xanthine and DL-valine, substrates for other potential peroxisomal oxidases, had no effect on chemiluminescence (not shown). Parish (1975), Hayashi and Suga (1978) and we (Fisher and Karampetsos, unpublished) failed to detect any xanthine oxidase or D-amino acid oxidase activity in *D. discoideum* amoebae. Xanthine oxidase activated during oxygen deprivation (ischaemia) is believed to contribute to post-ischaemic tissue damage in humans (Halliwell and Gutteridge, 1985).

Lipid peroxidation may play a role in chemiluminescence

Pharmacological evidence. In mammalian tissues the addition of cumene hydroperoxide enhances chemiluminescence by participating in lipid peroxidation reactions (Cadenas *et al.* 1984). We tested whether *D. discoideum* chemiluminescence was similarly enhanced by the addition of cumene hydroperoxide and found that it was, at concentrations of 200 μM and 2 mM (Fig. 14). Tenfold

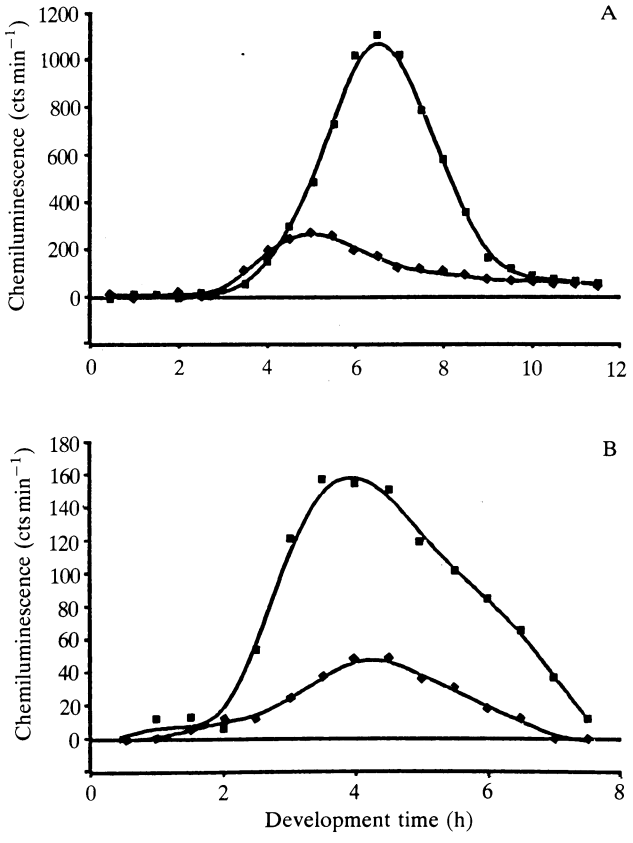

Fig. 13. Inhibition of chemiluminescence by the urate oxidase inhibitors 2,6,8-trichloropurine (A), and violurate (B). Each inhibitor was incorporated into the agar at a concentration of 0 (■) or 10 (◆) mM. Lower concentrations had no effect on chemiluminescence. Normal development in all 21 °C control vials indicated lack of cytotoxicity at all concentrations used. The difference in peak light emission between control vials in A and B is due to differences between batches of cells in different experiments. Other details as in Fig. 2.

higher concentrations totally inhibited chemiluminescence and were cytotoxic as judged by inhibition of development at 21 °C (Fig. 14). Two inhibitors of lipid peroxidation were tested: desferal, which works by removing the necessary free iron, and α-tocopherol (Vitamin E) which intercepts lipid peroxyl radicals (Cadenas *et al.* 1984). Desferal inhibited chemiluminescence as noted earlier, but α-tocopherol had no effect (not shown). If the light-emitting reactions occur at intracellular membranes away from the plasma membrane, it is possible that they were inaccessible to α-tocopherol, particularly since this compound is insoluble in water.

Spectral evidence. The known pathways for light emission in other chemiluminescent cells involve either production of singlet oxygen, which emits red and far red light on collapsing back to its ground state, or blue light emission from excited carbonyl radicals formed during lipid peroxidation (Cadenas *et al.* 1984). We measured the chemiluminescence emission and photodetector sensitivity spectra in our experiments by placing Kodak Wratten wavelength cutoff filters between the vial containing the amoebae and the photodetector. To measure the photodetector sensitivity spectrum we allowed white fluorescent light to leak into the photometer and used the same filters. The results showed that the

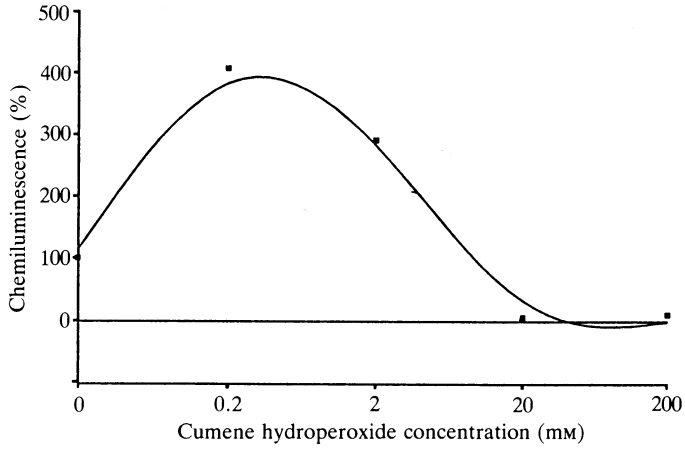

Fig. 14. Effect on chemiluminescence of cumene hydroperoxide, which stimulates lipid peroxidation. Cumene hydroperoxide was incorporated into the Tris–agar at the indicated concentrations. Development in the 21 °C control vials was normal at concentrations up to 2 mM, but was totally inhibited, indicating cytotoxicity, at the two higher concentrations used. Other details as for Fig. 1.

photodetector was insensitive to red light (λ>610 nm, Fig. 15B) and that most of the measured chemiluminescence was blue light (λ<450 nm, Fig. 15A). The ratio of the chemiluminescence spectrum (Fig. 15A) to the detector sensitivity spectrum (Fig. 15B) yielded a rough emission spectrum (Fig. 15C). Chemiluminescent *Dictyostelium* amoebae emit blue light at wavelengths shorter than 450 nm but very little in the 450 nm to 550 nm range (Fig. 15C). A small amount of light at λ>550, where the photodetector rapidly became insensitive, suggests that, as well as emitting blue light, the amoebae may emit light at longer wavelengths not detected by our equipment. Our results thus do not exclude light emission from singlet oxygen, but they are consistent with a role for lipid peroxidation in *Dictyostelium* chemiluminescence. In other experiments we were unable to inhibit chemiluminescence with any of the O_2^1 traps, β-carotene, crocetin and L-carnosine (not shown). We conclude that the chemiluminescence measured by our equipment does not involve singlet oxygen and probably results from lipid peroxidation reactions.

Discussion

Eukaryotic cells contain multiple oxidases in several intracellular compartments where O_2^-, H_2O_2 and thence $OH^\cdot$ might be generated. The chemical agents used in this work suggest at least two possible pathways leading to light emission (Fig. 16). The first of these involves leakage of electrons from ubiquinone in mitochondrial electron transport to molecular oxygen leading to O_2^- production. A role for this pathway is supported by the effects on chemiluminescence of rotenone, antimycin A and dibromothymoquinone. The second involves peroxisomal urate oxidation with production of H_2O_2. This pathway is supported by the effects on chemiluminescence of urate, violurate and 2,6,8-trichloropurine, and by the observation of Hayashi and Suga (1978) that urate oxidase and catalase activity in *Dictyostelium* increase after 4 to 6 hours of starvation. It is not supported by our inability so

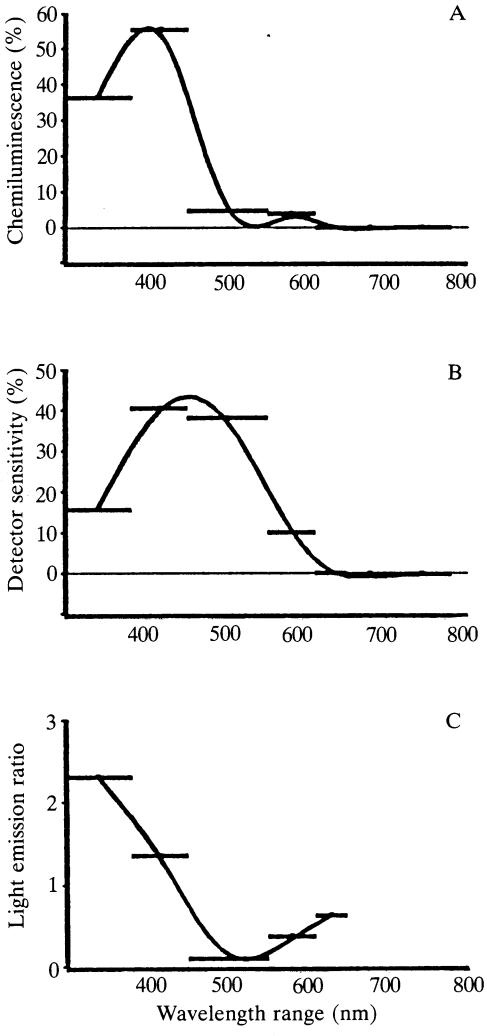

Fig. 15. *D. discoideum* chemiluminescence, photodetector sensitivity and light emission spectra. Kodak Wratten cutoff filters were placed between the photodetector of the New Brunswick ATP Photometer and the light source, which was either chemiluminescent *D. discoideum* amoebae (A) or white fluorescent light allowed to leak into the photometer (B). The difference between the amount of light passed by filters with successive wavelength cutoffs was calculated as a percentage of the total light measured in the absence of any filter. The spectral ranges for which these proportions of the total light apply are indicated by the horizontal bars. The values for the bar extending from the vertical axis to 350 nm represent light at all wavelengths below 350 nm, as no filters with shorter wavelength cutoffs than 350 nm were used. The ratio of the chemiluminescence spectrum in A to the detector sensitivity spectrum in B yields the rough emission spectrum in C. Ratios for the two highest wavelength ranges in A and B could not be calculated as the detector was totally insensitive at these wavelengths.

far to detect urate oxidase activity in bacterially grown chemiluminescent amoebae, although we do find the enzyme activity in axenically grown cells at similar levels to those reported by Parish (1975) (Fisher and Karampetsos, unpublished).

Whether the initial reduction of molecular oxygen takes place at the mitochondrial membrane or in peroxisomes, the interconversion of O_2^-, H_2O_2 and $OH^\cdot$ follows spontaneously and in iron- and SOD-catalysed reactions, while light emission results from both lipid peroxidation and O_2^1 production (Fig. 16). These conclusions are supported by developmental changes in the activities of SOD (Brewster and Wheldrake, 1989) and catalase (Hayashi and Suga, 1978) and by the effects on chemiluminescence of phenazine methosulphate, diethyl carbamate, SOD, H_2O_2, catalase, mannitol, glycerol, allopurinol, histidine, desferal and cumene hydroperoxide. With our equipment blue light presumably associated with lipid peroxidation was the major contributor to the measured chemiluminescence.

In leukocytes chemiluminescence results from the activity of a plasma membrane NADPH oxidase, is inhibited by catalase and SOD and is enhanced by the addition of luminol, which intercepts oxygen free radicals in light-emitting reactions (Cadenas *et al.* 1984). Luminol-enhanced chemiluminescence depends on O_2^- (Merenyi *et al.* 1985), and we found light emission by *D. discoideum* amoebae to be unaltered by luminol at concentrations up to 100 μM (unpublished data). This suggests that luminol may be unable to penetrate the cell membrane and that short-lived superoxide anions do not escape the cell in significant quantities. In that case, the enhancement of chemiluminescence by SOD would be due to its light-yielding reaction with H_2O_2, rather than to enhanced production of H_2O_2 from O_2^-.

Only H_2O_2 of the various O_2 metabolites would be sufficiently long-lived and able to permeate membranes to pass out of the cell, if the initial O_2 reduction to O_2^- takes place at an intracellular location away from the plasma membrane. This would distinguish *Dictyostelium* chemiluminescence from that found in leukocytes and could explain why catalase in the medium inhibits, SOD enhances and luminol has no effect on chemiluminescence. A plasma membrane-bound NADPH oxidase such as exists in leukocytes seems unlikely to be the source of reduced oxygen metabolites and chemiluminescence in *Dictyostelium*. Chemiluminescence in this organism seems more akin to that found in mammalian cells other than leukocytes.

We previously showed that chemiluminescence in *D. discoideum* is both developmentally and heat-shock-regulated (Fisher and Rosenberg, 1988). Given that development itself is initiated in this organism by nutritional stress (starvation), this suggests a link between stress responses in cells and perturbed oxidative metabolism. Possibly heat shock causes oxidative stress, which in turn induces heat shock protein synthesis and production of reduced O_2 metabolites. Consistent with such a scenario is the finding that many of the agents that stimulate chemiluminescence in mammalian cells cause oxidative stress and also induce the synthesis of heat shock proteins (Leenders *et al.* 1974; Anderson, 1989).

The major heat shock protein of eukaryotes, hsp70, is now known to function in transport of proteins across cellular membranes, in particular the mitochondrial membrane (Lindquist and Craig, 1988). In *D. discoideum* hsp70 is also the major heat shock protein and is synthesized maximally after heat shock at a time that corresponds with the peak chemiluminescence (Loomis and Wheeler, 1980; Zhao and Fisher, unpublished). This fits well with a possible mitochondrial origin for oxy-radicals involved in stress-induced chemiluminescence in *Dictyostelium*.

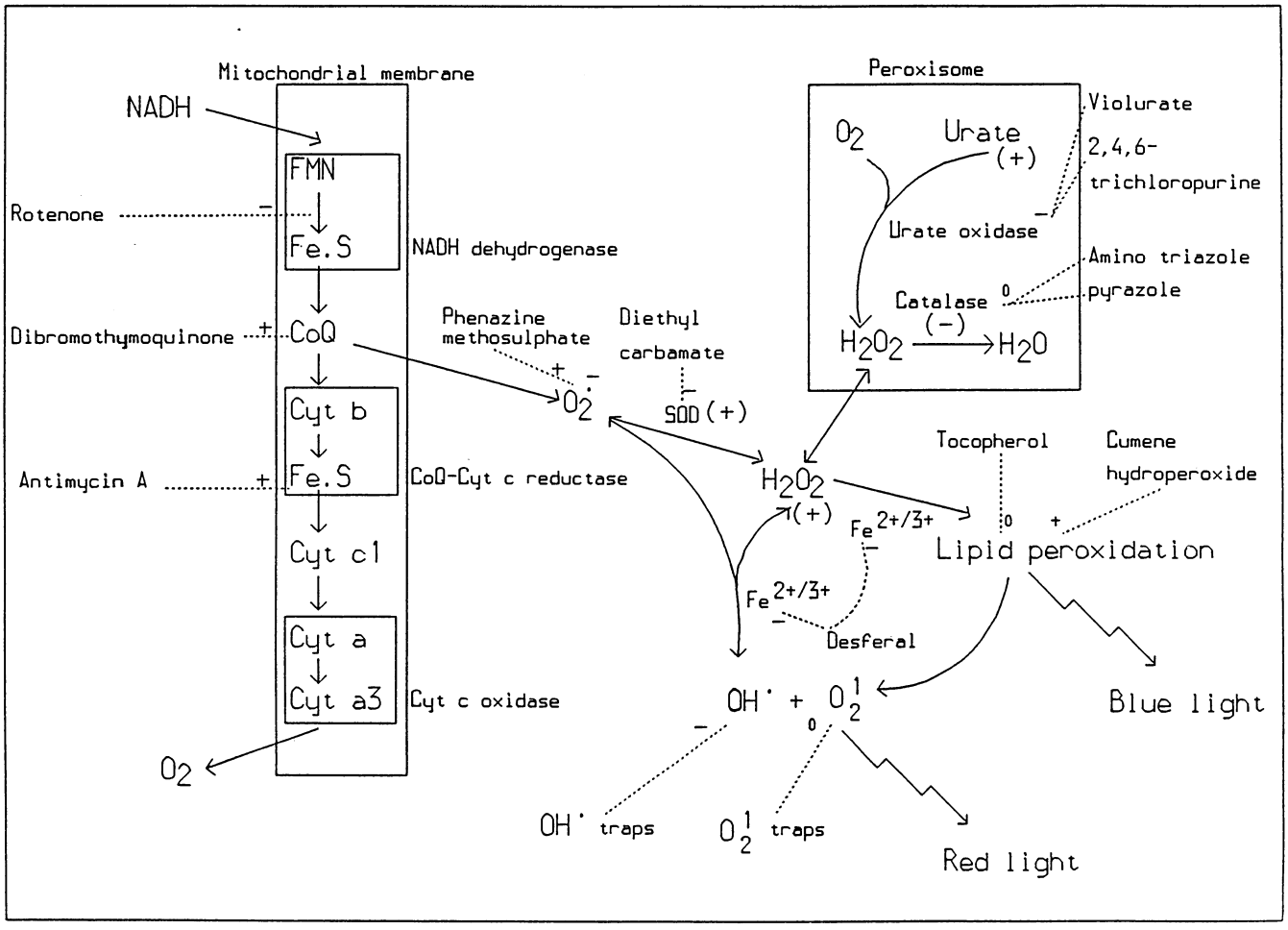

Fig. 16. Possible pathways for chemiluminescence in *Dictyostelium discoideum*. Reduced oxygen metabolites could be generated by one or both of two pathways: leaky mitochondrial electron transport producing O_2^- and peroxisomal oxidation of urate producing H_2O_2. (.....$^+$.) indicates the site of action of a chemical agent that enhanced chemiluminescence; (.....$^-$.) indicates the site of action of a chemical agent that inhibited chemiluminescence; (.....^{0}.) indicates the expected site of action of a chemical that had no effect on chemiluminescence; (+) indicates a participant in the pathways that enhanced chemiluminescence when added to the agar; (−) indicates a participant in the pathways that inhibited chemiluminescence when added to the agar. SOD is superoxide dismutase.

We are grateful to Mr J. Monastirli, Mr W. Zhao and Ms H. Kasimiotis for assistance with some of the chemiluminescence experiments. This work was supported by a grant from the Australian Research Council.

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(Received 15 March 1991 – Accepted 30 April 1991)