Chemiluminescence of *Acanthamoeba castellanii*

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1. Chemiluminescence of *Acanthamoeba castellanii* in the presence of O_2 was of similar intensity in organisms harvested early or late during exponential growth [when cyanide (1 mM) stimulates or inhibits respiration respectively]. 2. Cyanide (up to 1.5 mM) stimulated photoemission in both types of organism by 250–300 photons/s per 10^7 cells above the value observed under aerobic conditions. 3. 'Dibromothymoquinone' (2,5-dibromo-6-isopropyl-3-methyl-*p*-benzoquinone) (up to 80 μM) further increased chemiluminescence. 4. Similar responses were also demonstrated in whole homogenates and in subcellular fractions; 36% of the chemiluminescence was provided by a fraction sedimenting at 100000*g*-min, and 20% in that fraction that was non-sedimentable at 200000*g*-min. 5. Mitochondrial substrates (succinate, 2-oxoglutarate, NADH) in the presence or absence of ADP and P_i or peroxisomal substrates (glycollate, urate or ethanol) gave no increases in light emission by whole homogenates or in any of the fractions. 6. It is suggested that reactions responsible for production of chemiluminescence are those primarily producing superoxide anions and leading to lipid peroxidation and singlet-oxygen formation. Photoemission enhancement and superoxide dismutase inhibition showed similar cyanide concentration-dependencies.

A weak ('low-level') chemiluminescence has been detected from mouse liver, muscle and brain (Tarusov *et al.*, 1961, 1962), and in yeast (Stauff & Reske, 1964; Esser & Stauff, 1968; Quickenden & QueHee, 1974). Studies with rat liver microsomal fractions suggested that reactions responsible for this chemiluminescence include oxidative chain reactions producing singlet oxygen, hydroxy radicals and lipoperoxides (Pyatenko & Tarusov, 1964; Howes & Steele, 1971, 1972; Nakano *et al.*, 1976; Sugioka & Nakano, 1975). Rat liver mitochondria also produce a weak chemiluminescence (Boveris *et al.*, 1979). Intermediary stages in the reduction of molecular oxygen, for instance in the ubiquinone–cytochrome *b* region of the respiratory chain, yield superoxide anions and hence hydrogen peroxide by way of superoxide dismutase (Loschen *et al.*, 1971; Boveris *et al.*, 1972; Loschen *et al.*, 1974; Boveris & Cadenas, 1975), probably at the redox level of ubisemiquinone (Boveris *et al.*, 1976; Cadenas *et al.*, 1977). Superoxide generation in plant mitochondria also occurs at the flavoprotein region of the NADH dehydrogenase segment of the respiratory chain (Rich & Bonner, 1978). The primary generation of super-

oxide anions and hydrogen peroxide may thus be linked to chain reactions leading to lipid peroxidation (Chance & Boveris, 1978) and to the production of chemiluminescent species in a number of membrane redox systems.

The soil amoeba *Acanthamoeba castellanii* has a branched mitochondrial electron-transport chain, terminating in three distinct oxidases (Edwards *et al.*, 1977; Edwards & Lloyd, 1977, 1978*a*,*b*). The main respiratory chain terminates in cytochrome $a+a_3$, which is azide- and cyanide-sensitive. A second oxidase is azide- and cyanide-insensitive but is inhibited by salicylhydroxamic acid. The third oxidase is insensitive to azide and salicylhydroxamic acid but is inhibited by cyanide. Dibromothymoquinone, acting as an analogue of ubiquinone, inhibits mitochondrial electron transport at the ubiquinone–cytochrome *b* region of the respiratory chain (Trebst *et al.*, 1970); in *A. castellanii* this compound decreases electron flux through both the azide- and salicylhydroxamic acid-sensitive pathways but not that through the third oxidase (S. W. Edwards & D. Lloyd, unpublished work). The relative electron flux through these oxidases changes as organisms grow in a batch culture; organisms harvested in early phase of exponential growth show cyanide-stimulated respiration, and in these organisms respiration can proceed by way of two pathways alternative to the main phosphorylating chain (Lloyd *et al.*, 1979). In late-exponential-phase

cultures the pathway of electron transport is to a greater extent by way of a conventional cyanide- and azide-sensitive cytochrome c oxidase having a high affinity for oxygen.

In the present paper we report that the intensity of photon emission in *A. castellanii* is similar both in organisms with cyanide-stimulated and with cyanide-inhibited respiration; chemiluminescence is observed in four subcellular fractions and is enhanced by cyanide alone or by cyanide plus dibromothymoquinone.

Experimental

Maintenance and growth of the organism

Acanthamoeba castellanii was maintained and grown axenically with shaking at 30°C exactly as described previously (Edwards *et al.*, 1977). Organisms were counted in a Fuchs–Rosenthal haemocytometer slide (Baird and Tatlock, Chadwell Heath, Romford, Essex, U.K.) after suitable dilution with 50 mм-MgCl$_2$.

Harvesting of cultures, cell disruption and subcellular fractionation

Cultures were harvested by centrifugation at 100 g for 1.5 min in the 6×250 ml rotor of a Sorvall RB2 centrifuge. Organisms were washed twice in 50 mм-MgCl$_2$, pH 6.0, and resedimented. Resuspension in 3 vol. of 50 mм-MgCl$_2$ provided the cell suspensions for experiments with non-proliferating whole cells. For subcellular fractionation, the organisms were resuspended in 0.32м-sucrose/10 mм-Tris/H$_2$SO$_4$/0.2 mм-EGTA, pH 8.0. Disruption was by hand in a glass homogenizer with Teflon pestle (A. H. Thomas, P.O. Box 779, Philadelphia, PA, U.S.A.); six strokes were adequate for about 90% cell breakage. Remaining whole cells together with nuclei were removed by centrifugation at 1000 g ($r_{av.}$ 7.5 cm) for 8 min in the 8×50 ml rotor of the Sorvall RB2 centrifuge. The supernatant ('cell-free extract') was carefully removed and centrifuged at 10000 g ($r_{av.}$ 7.6 cm) for 10 min. The sediment was resuspended in 5 vol. of the disruption buffer and resedimented at 10000 g ($r_{av.}$ 7.6 cm) for 10 min to
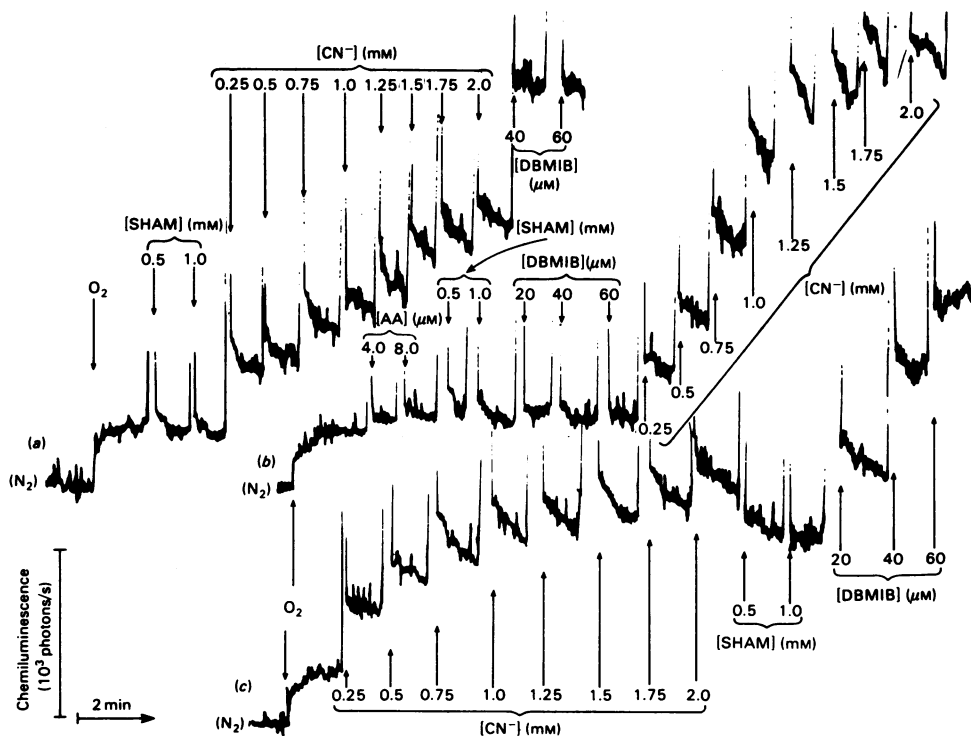

Fig. 1. *Chemiluminescence of intact-cell suspensions of early-exponential-phase A. castellanii*
Organisms were harvested at a cell density of 3.1×10^5 cells/ml and resuspended at 4.4×10^7 cells/ml in 50 mм-MgCl$_2$. Abbreviations used: SHAM, salicylhydroxamic acid; DBMIB, dibromothymoquinone; AA, antimycin A. (*a*), (*b*) and (*c*) represent three traces of chemiluminescence.

give a pellet (P_1) and supernatant (S_1); the post-mitochondrial supernatant was centrifuged at 20000g for 10min to yield a pellet (P_2) and a final supernatant (S_2). P_2 was resuspended in 5vol. of disruption buffer.

Measurement of chemiluminescence

The single-photon counting utilized a 5cm-end-window photomultiplier (8850, RCA Electronic Components, Harrison, NY 07029, U.S.A.) responsive in the spectral range 300–650nm, with an applied voltage of 1.8kV, connected to an amplifier–discriminator (Princeton Applied Research, P.O. Box 2565, Princeton, NY 08540, U.S.A.) adjusted for single photons. The output was monitored with a frequency counter (Heath IB1110, Heath Co., Benton Harbor, MI 49022, U.S.A.) and a potentiometric recorder. The photomultiplier was sealed into an Ortec housing and surrounded by solid CO_2; the tube temperature was approx. $-30°C$. A Lucite (Perspex) rod (22cm long, 5cm diam.) enclosed in aluminium foil served as optical coupler and also ensured thermal isolation from the sample. Suspensions (4.5ml) were held in a Lucite cuvette (35mm × 35mm × 5mm, 12.25cm^2 surface area) placed in front of the optical coupler and maintained at 30°C. Gassing of the cuvette contents was via two stainless-steel microtubes (gauge no. 24) placed at the bottom corners of the cuvette. A plan of the apparatus has been published elsewhere (Boveris *et al.*, 1979).

Other analytical procedures

Dissolved oxygen concentrations were measured simultaneously with chemiluminescence by using a Teflon-covered Clark electrode in a specially designed cuvette (total vol. 9ml). Cytochrome c oxidase (EC 1.9.3.1) activity was measured by the method of Smith (1955), catalase (EC 1.11.1.6) was determined as described by Lück (1963), and superoxide dismutase (EC 1.15.1.1) as described by Boveris & Stoppani (1977). Protein was assayed by the pro-
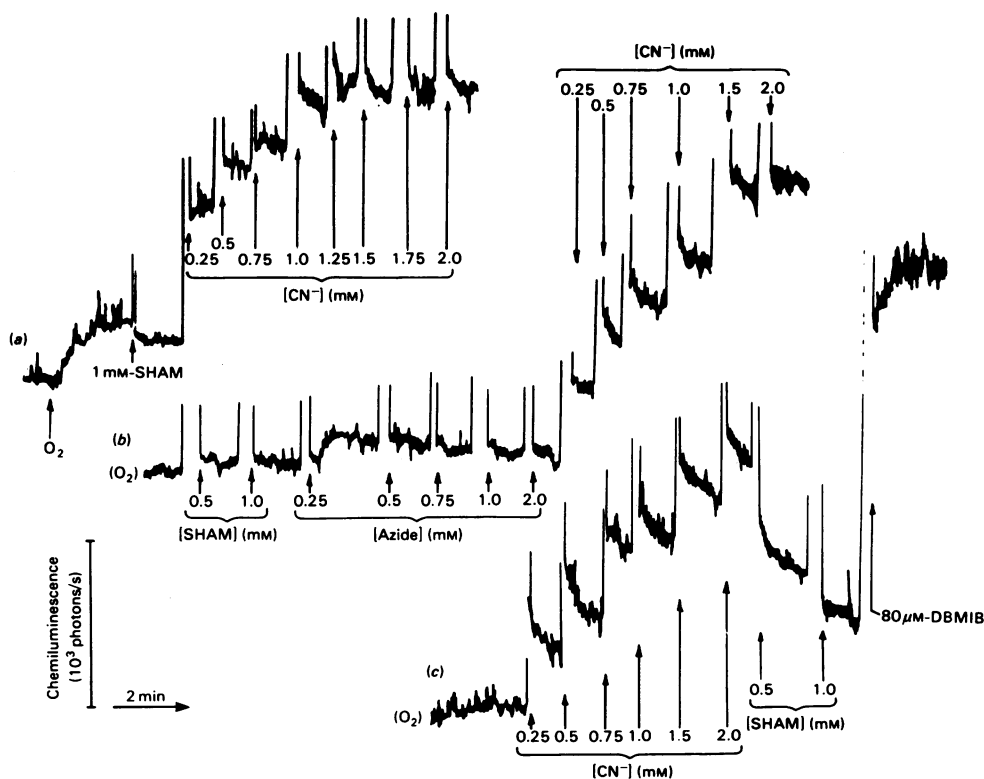

Fig. 2. *Chemiluminescence of intact-cell suspensions of late-exponential-phase A. castellanii*
Organisms were harvested at a cell density of 1.7×10^6 cells/ml and resuspended to 4×10^7 cells/ml in 50mm-$MgCl_2$. Abbreviations used: SHAM, salicylhydroxamic acid; DBMIB, dibromothymoquinone. (*a*), (*b*) and (*c*) represent three traces of chemiluminescence.

cedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chemicals

Cytochrome *c* (type III, from horse heart) NADH (type III, from yeast), ATP, ADP, xanthine oxidase, adrenaline (epinephrine), antimycin A, sodium glycollate and sodium urate were all from Sigma. t-Butyl hydroperoxide and dimethylfuran were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. 'Dibromothymoquinone' (2,5-dibromo-6-isopropyl-3-methyl-*p*-benzoquinone) was from Dr. W. Draber, Bayer A.G. BRD 56 Wuppertal, Germany.

Results

Chemiluminescence of whole-cell suspensions

Light emission by organisms from an early-exponential-phase culture is shown in Fig. 1. An anaerobic suspension of organisms gave a count of 1100 photons/s. Oxygenation increased the intensity of chemiluminescence by 500 photon/s. Salicylhydroxamic acid (1 mM) gave no response, but sequential additions of cyanide (0.25–2 mM in eight equal steps) produced a considerable enhancement of light-emission (Fig. 1*a*). A further increase in light-intensity was seen on adding 40 μM-dibromothymoquinone. Antimycin A (up to 8 μM) produced no significant increase in chemiluminescence (Fig. 1*b*), neither did subsequent additions of 1 mM-salicylhydroxamic acid and dibromothymoquinone (60 μM). Cyanide (up to 2 mM) increased chemiluminescence when added last in this sequence. Fig. 1(*c*) shows that cyanide alone also increased the response; salicylhydroxamic acid added after cyanide gave no effect. The declining signals in the presence of cyanide were due to loss of the inhibitor from the oxygenated

system at the pH used (pH 6.0). Dibromothymoquinone (up to 60 μM) added after cyanide and salicylhydroxamic acid again gave elevated responses. Quenching of the chemiluminescence (results not shown) occurred on adding 15 mM-dimethylfuran.

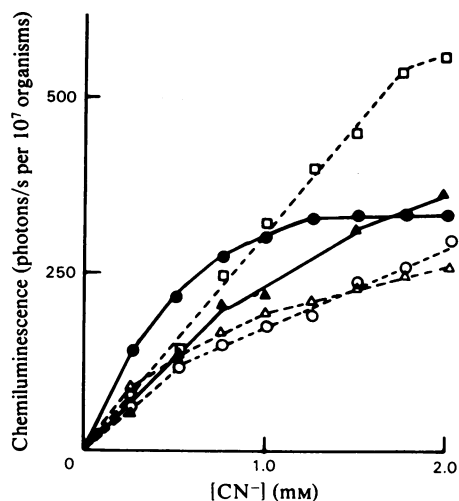As the response to inhibitors of respiration of organisms from the early- and late-exponential

Fig. 3. *Effects of respiratory inhibitors on the intensity of photon emission by whole-cell suspensions of A. castellanii*
Open symbols, early-exponential-phase organisms; closed symbols, late-exponential-phase organisms. ▲, △, Cyanide alone; ●, ○, salicylhydroxamic acid (1 mM) added before cyanide; □, salicylhydroxamic acid (1 mM) and dibromothymoquinone (60 μM) added before cyanide. The zero value on the ordinate represents photon emission in the oxygenated suspensions.
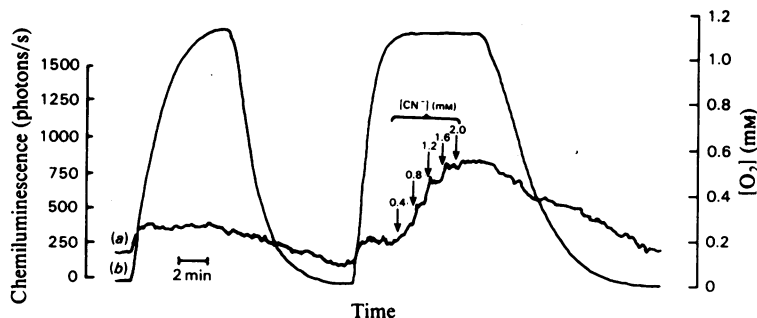
Fig. 4. *Chemiluminescence of whole-cell suspension of A. castellanii: responses to oxygenation and anaerobiosis*
Organisms were harvested in late-exponential phase of growth and resuspended in 50 mM-MgCl₂ (pH 6.0) at 3.1 mg of protein/ml. Additions of cyanide were made where indicated by the arrows. (*a*), Chemiluminescence; (*b*), [O₂].

phases of growth are quite different (see the introduction), a similar experiment was performed with organisms harvested at a later stage in growth. Fig. 2 shows that cyanide in the presence (Fig. 2a) or absence (Fig. 2c) of salicylhydroxamic acid produced a similar elevation of emitted light-intensities. Azide (up to 2mM) gave no response in the presence of salicylhydroxamic acid, and dibromothymoquinone (80μM) added after cyanide gave a large response. The final intensities of emission from organisms in the presence of 2mM-cyanide in the late-exponential phase of growth was about 25% higher than that from organisms harvested earlier in growth (Fig. 3). This intensity of emission was not increased by the presence of salicylhydroxamic acid. The concentration of cyanide giving 50% stimulation of light emission from whole cells was around 0.7mM.

Dependence of chemiluminescence on the presence of O_2 is shown in Fig. 4; switching from O_2 to N_2

gave a transition to anaerobiosis, which, as indicated by the polarographic trace, was complete in 6min. However, the accompanying decrease in light-intensity commenced immediately, whereas the dissolved oxygen concentration was still > 1.0mM. A return to O_2 produced a rapid re-establishment of light intensity. When 2mM-cyanide was added to the oxygenated suspension, then N_2 passed in, the decay of chemiluminescence was biphasic (Fig. 4). In the presence of cyanide the return to oxygenation from anaerobiosis produced an overshoot in photo-emission (Fig. 5); this phenomenon was observed in both cyanide-stimulated and cyanide-inhibited cell types. The dependence of chemiluminescence on O_2 concentration in the presence and absence of 2mM-cyanide for both cell types (Fig. 6) indicates the presence of two components, one with a high O_2-concentration-dependence, and a second still functional at lower O_2 partial pressures.
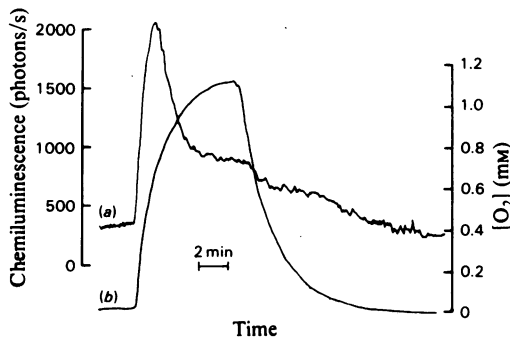

Fig. 5. *Overshoot of luminescence during oxygenation of whole-cell suspension of A. castellanii in the presence of cyanide*

Conditions were as described in Fig. 4. The concentration of cyanide was 2mM. (a) Chemiluminescence; (b), [O_2].
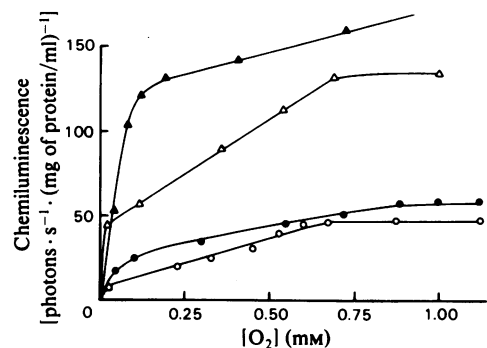

Fig. 6. *Effect of oxygen concentration on chemiluminescence of A. castellanii*

Early-exponential-phase organisms: △, 2mM-cyanide; ○, without cyanide. Late-exponential-phase organisms: ▲, 2mM-cyanide; ●, without cyanide.
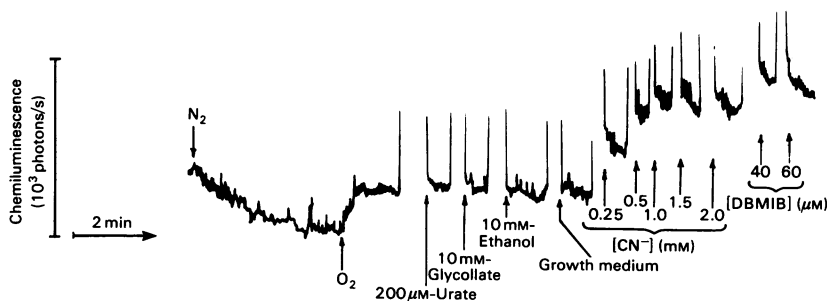

Fig. 7. *Chemiluminescence of cell-free extract of A. castellanii*

A cell-free extract (4.6mg protein/ml) was prepared from organisms harvested in the late-exponential phase of growth (2×10^6 cells/ml). Abbreviation used: DBMIB, dibromothymoquinone.
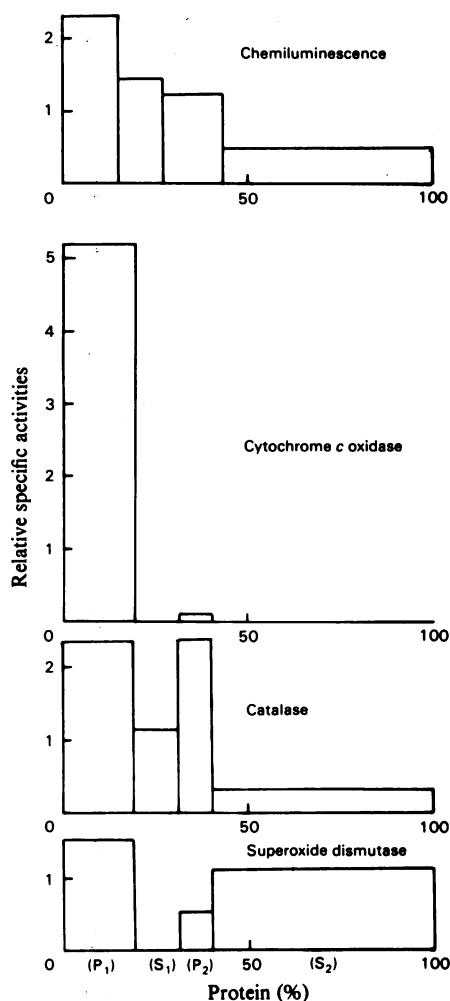
Fig. 8. *Distribution of enzymes and photoemission (in the presence of 2 mM-cyanide) in cell-free extracts of A. castellanii after differential centrifugation*

Relative specific activities (the ratios of specific activities in fractions to those in the cell-free extract) were plotted against cumulative percentage of protein recovered in each fraction. The centrifugal field increases from left to right. The far-right-hand bar represents the fraction containing particles non-sedimentable at 200000g-min. Percentage recoveries · (based on enzyme units in the cell-free extracts) were 99.4% for chemiluminescence, 90.1% for cytochrome c oxidase, 78% for catalase, 130% for superoxide dismutase, and 118% for protein respectively. Chemiluminescence and enzyme distributions were determined in separate fractionations.

Chemiluminescence of cell-free extracts and fractions

Chemiluminescence of the cell-free extract (Fig. 7) showed some characteristics similar to those described

previously for whole cells. Anaerobic–aerobic cycles of light-emission could be demonstrated, and the greatest responses were produced on adding cyanide and dibromothymoquinone. Addition of peroxisomal substrates (urate, glycollate or ethanol) gave no effect. Addition of growth medium (1.5% glucose/0.75% yeast extract/0.75% proteose–peptone) was also without effect.

Subcellular fractionation by differential centrifugation gave four fractions, P_1, S_1, P_2, and S_2 (Fig. 8). Distribution of light-emission (in the presence of 2 mM-cyanide) among these fractions was compared with the distribution of cytochrome c oxidase (mitochondrial marker enzyme), catalase (up to 98% peroxisomal; Morgan et al., 1973) and superoxide dismutase. Fraction P_1 contained 15% of the protein, 99% of the cytochrome c oxidase, 45% of the catalase, 29% of the superoxide dismutase, and accounted for 35% of the total chemiluminescence. Fraction S_1 contained 12% of the protein, 14% of the catalase and gave 17% of the total photoemission. Fraction P_2 contained 16% of the protein, 21% of the catalase and gave 20% of the photoemission. Fraction S_2 contained 57% of the protein, 20% of the catalase, 66% of the superoxide dismutase and accounted for 30% of the chemiluminescence.

No stimulation of photoemission was observed in any fraction by urate, glycollate, ethanol or growth medium, but all four fractions responded to addition of cyanide or cyanide-plus-dibromothymoquinone. Mitochondrial fractions from organisms harvested in early- or late-exponential phases of growth gave no increased photoemission over that observed under aerobic conditions on addition of succinate, NADH or 2-oxoglutarate in the presence or absence of ADP. Addition of t-butyl hydroperoxide gave a doubling of chemiluminescence intensity irrespective of previous additions. Chemiluminescence of cell-free extracts and subcellular fractions was slightly more sensitive to stimulation by cyanide than that of intact organisms (Fig. 9a); cyanide-dependencies for inhibition of cellular respiration, cytochrome c oxidase, catalase and superoxide dismutase activities in a whole homogenate of A. castellanii are shown (Fig. 9b). Cellular respiration, cytochrome c oxidase and catalase were all more sensitive to inhibition by cyanide (K_i values 60, 60 and 140 μM respectively) than was stimulation of chemiluminescence by cyanide, but superoxide dismutase was half-inhibited at a concentration similar to that giving 50% of total cyanide-induced photoemission in cell-free extracts (0.3 mM).

Discussion

Acanthamoeba castellanii shows a 'low-level' chemiluminescence (as defined by Seliger, 1975)
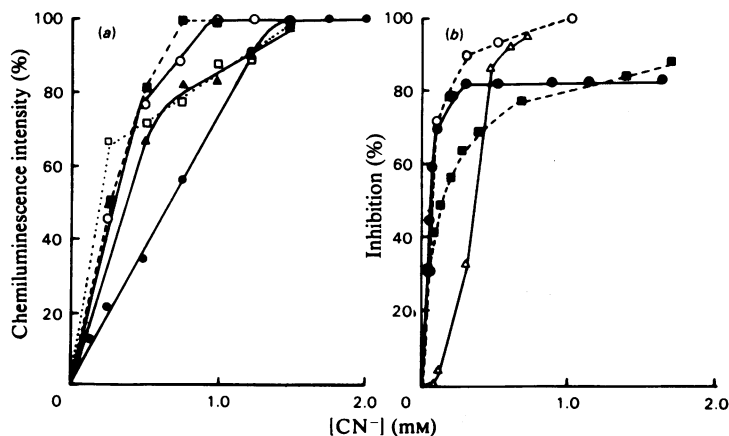
Fig. 9. *Effect of cyanide on chemiluminescence of intact-cell suspensions, subcellular fractions and enzymes of A. castellanii* (a) Cyanide stimulation of chemiluminescence (percentage of maximum intensity produced on cyanide addition) in: ●, intact late-exponential-phase cells resuspended at 2.7×10^7/ml; ○, whole homogenate (4.6mg protein/ml); □, mitochondrial fraction (1.8mg protein/ml); ■, fraction sedimented at 200000g-min (110.5mg of protein/ml); ▲, fraction non-sedimentable at 200000g-min (6.8mg of protein/ml). (b) Cyanide-sensitivity of: ●, respiration of: whole cells: ○, cytochrome c oxidase; ■, catalase; and △, superoxide dismutase, in a whole homogenate of late-exponential-phase organisms (0.2mg of protein/ml).

that is dependent on the presence of molecular oxygen, and its O_2-concentration-dependence suggests the presence of two components. Its intensity is similar in organisms harvested early or late in exponential growth, even though the proportions of mitochondrial electron transport through the main phosphorylating chain and the two alternative oxidases differ markedly in the two cell types (Edwards & Lloyd, 1978a,b). Thus the phenomenon of light-emission is not directly correlated with the extent of reduction of oxygen at respiratory sites other than cytochrome c oxidase. Furthermore, cyanide specifically enhances photon emission in both cell types, whereas azide or antimycin A (both inhibitors of the main respiratory chain in *A. castellanii*) do not. In isolated mitochondria, succinate, 2-oxoglutarate or NADH additions made before cyanide do not increase the response of photon emission to this inhibitor.

Subcellular-fractionation experiments implicate a multiplicity of locations in photoemission; fractions containing mitochondria, peroxisomes and the microsomal fractions all exhibit cyanide-stimulated photoemission. Peroxisomal substrates (glycollate, urate and ethanol), which generate H_2O_2 in *A. castellanii*, did not produce increased chemiluminescence.

Enzymes sensitive to inhibition by cyanide in *A. castellanii* include cytochrome c oxidase, catalase and superoxide dismutase. The concentration-dependence of stimulation of photoemission by cyanide resembles most closely the sensitivity of superoxide dismutase to this inhibitor; moreover,

this enzyme activity is multilocational in subcellular fractions. It seems likely that the remarkable effect of cyanide on chemiluminescence is mediated via increased intracellular superoxide anion concentration, which may in turn increase the generation rate of hydroxy radicals (McCord & Day, 1978; Halliwell, 1978), leading to lipid hydroperoxide and singlet-oxygen formation (Nakano *et al.*, 1975; Sugioka & Nakano, 1976). Singlet oxygen is itself chemiluminescent (Khan & Kasha, 1963; Kearns, 1971).

The enhancement of *A. castellanii* chemiluminescence by t-butyl hydroperoxide, as previously observed in rat liver mitochondria and microsomal fractions (Boveris *et al.*, 1979), seems to indicate that a lipid hydroperoxide (RO_2H) and superoxide anions may afford the reactants that directly or indirectly lead to the formation of chemiluminescent species; recent evidence implicates haemoproteins in this process (E. Cadenas, personal communication).

Anaerobiosis in the presence of cyanide allows pools of intermediates to accumulate that give a burst of chemiluminescence on admission of O_2. Possible candidates include ubisemiquinone (Boveris *et al.*, 1976; Cadenas *et al.*, 1977) and a reduced form of an autoxidizable cytochrome b (Loschen *et al.*, 1973). The action of dibromothymoquinone requires further investigation, but it is noteworthy that this inhibitor enhances chemiluminescence only when cyanide is present. That this quinone analogue produces an effect in all subcellular fractions means that its action is not restricted to a site at the ubiquinone–cytochrome b areas of the respiratory chain

(Trebst *et al.*, 1970). It seems likely that dibromo-thymoquinone, similarly to other benzo- and naphtho-quinones (Boveris *et al.*, 1976, 1978), could be reduced by different intracellular redox systems and then originate superoxide anion by autoxidation.

Chemiluminescence seems to afford an integrative measurement of reactions closely related to lipid and organic peroxide formation. It offers unique advantages as a non-destructive assay with high sensitivity, since light-emission enables the detection of 100 am–10 fM concentrations of the photoemissive species.

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