Chemiluminescence Induced by Operation of Iron-Flavoproteins*

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Chemiluminescence in the system xanthine oxidase + xanthine + 10,10′-dimethyl-9,9′-biacridylium nitrate (DBA^{++}) is a consequence of the two-electron reduction of DBA^{++} and the one-electron reduction of O_2 by the reduced enzyme. The reduced DBA and oxygen radical, so formed, react to form an excited DBA^{++} derivative which emits light as it returns to the ground state.

As reported by Totter *et al.* (1959, 1960a) the action of xanthine oxidase on its substrates in the presence of oxygen and dimethyl biacridylium (DBA^{++})[1] results in the emission of light. The structures of these and related compounds are shown in Figure 1. Involvement of oxygen radicals in the reactions leading to light emission has been postulated by Totter *et al.* (1960b). Our continuing interest in the production of oxygen radicals by oxidative enzymes (Fridovich and Handler, 1958, 1961, 1962) and certain unexplained kinetic peculiarities of these luminescent reactions has led to a reinvestigation of these phenomena. A preliminary communication of these findings has been reported (Greenlee *et al.*, 1961).

EXPERIMENTAL

Intensity of luminescence was measured with a Farrand Model A fluorometer, operated as a photometer. Reactions were performed in matched 10 × 75 mm Pyrex tubes, in a final volume of 1.0 ml, buffered at *p*H 10.0 by 0.10 N carbonate or 0.05 M glycinate. Spectrophotometric studies were performed with a Zeiss PM Q II spectrophotometer, equipped with an MM 12 double monochromator, or with a Cary Model 14 spectrophotometer. All reactions were conducted at room temperature and atmospheric pressure. Gas mixtures were prepared as previously described (Fridovich and Handler, 1962). *N*-methyl acridone, prepared according to Bergmann and Blum-Bergmann (1930), was converted to DBA° and DBA^{++} as described by Decker and Petsch (1935). The DBA^{++} thus obtained had a millimolar extinction coefficient of 8.7 at 430 mμ and of 34.0 at 368 mμ. Although not in complete agreement with corresponding values cited in the literature (Totter *et al.*, 1960a; Karyakin, 1952), these values were in accord with those obtained with a sample of DBA^{++} purchased from the

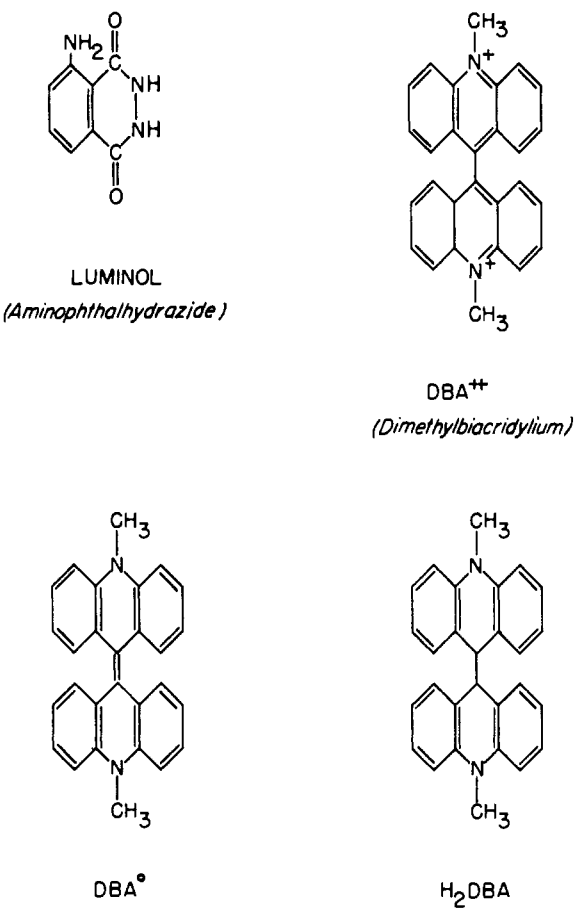

FIG. 1.—Structures of Luminol and of dimethylbiacridylium and its reduction products.

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[1] Abbreviations used: EDTA, ethylenediamine tetraacetate; Tiron, 1,2-dihydroxybenzene-3,5-disulfonate; DBA^{++}, 10,10′-dimethyl-9,9′-biacridylium nitrate; DBA°, 10,10′-dimethyl-Δ9,9′-biacridane; H_2DBA, 10,10′-dimethyl-9,9′-biacridane; Luminol, 5-amino-2,3-dihydrophthalazine-1,4-dione.

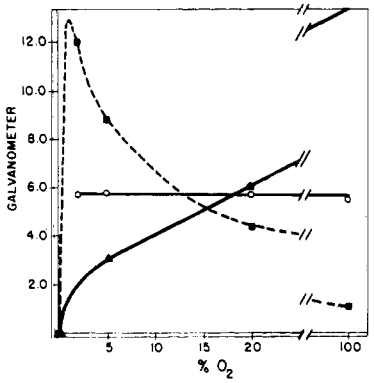

FIG. 2.—Effect of O_2 concentration on chemiluminescence induced by xanthine oxidase. The reaction mixture (■—■) contained Na_2CO_3, 0.05 N, *p*H 10.0; hypoxanthine, 10^{-4} M; DBA^{++}, 10^{-4} M; and 0.16 unit of xanthine oxidase in a total volume of 1.0 ml. H_2O_2 induced luminescence (○—○) was observed in a reaction mixture which contained Na_2CO_3, 0.1 N, *p*H 10.0; DBA^{++}, 10^{-4} M; and H_2O_2, 0.2 M. Enzymically induced luminescence of luminol (▲—▲) was observed in a system in which 2×10^{-3} M luminol replaced DBA^{++}.

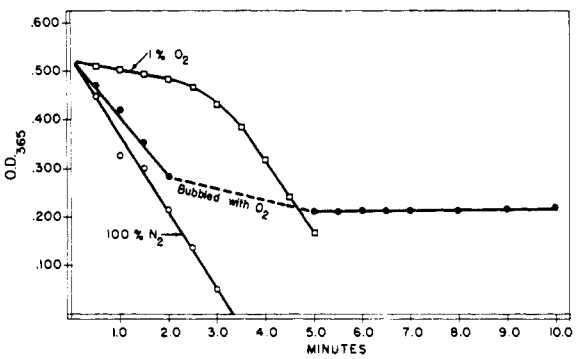

FIG. 3.—Inhibitory effect of O_2 on DBA^{++} reduction. Reaction mixture contained 0.08 N Na_2CO_3, *p*H 10.0, 10^{-4} M hypoxanthine, 3.3×10^{-5} M DBA^{++}, and 330 μg/ml bovine serum albumin in a total volume of 3.0 ml in an anaerobic cuvet with 0.1 unit of xanthine oxidase in the side-arm. After the mixture was gassed with N_2 for 15 minutes, enzyme was tipped in and OD at 365 mμ observed.

Chemicals Procurement Company of New York City, and with the results of stoichiometric reduction of DBA^{++} by xanthine plus xanthine oxidase under anaerobic conditions. Spectrophotometry under anaerobic conditions was performed in cuvets similar to those described by Lazarow and Cooperstein (1954). The oxidation of xanthine to uric acid at *p*H 10.0 was followed at 300 mμ; at this wave length the millimolar extinction coefficients are 1.3 and 10.5 respectively. The oxidation of hypoxanthine to xanthine under these conditions was followed at 281 mμ; at this wave length the millimolar extinction coefficient of hypoxanthine is 1.1 and xanthine and uric acid are isosbestic with coefficients of 8.9. Milk xanthine oxidase was prepared and its activity defined as described (Fridovich, 1962). All enzymic studies were conducted with xanthine oxidase except where indicated otherwise. Uricase (purified) and horseradish peroxidase (C grade) were obtained from Worthington Biochemical Corporation. Moccasin venom L-amino acid oxidase was prepared according to Singer and Kearney (1950). Cytochrome oxidase was a gift from Dr. D. E. Green.

RESULTS

Effect of Oxygen Concentration.—In systems containing DBA^{++}, chemiluminescence exhibited a complex dependence upon O_2 concentration. When air was continuously bubbled through the reaction mixture, light emission lasted six times as long as when chemiluminescence was observed in a closed system. When O_2 was maintained at constant concentration, the limiting factor was the substrate, even though the latter was present in more than 2-fold excess over DBA^{++}. Under anaerobic conditions, no light emission was observed. The effect, on the intensity of DBA^{++} luminescence, of continually gassing the reaction mixture with known mixtures of O_2 and N_2 is shown in Figure 2. Although essential for luminescence, O_2 in excess of approximately 2% of the gas phase was inhibitory. The failure of O_2 to affect the luminescence of DBA^{++} caused by H_2O_2 indicates that this inhibition by O_2 is not due to quenching.

Under anaerobic conditions, DBA^{++} was reduced by xanthine oxidase plus xanthine or hypoxanthine. This was conveniently followed at 365 mμ (Fig. 3). At a partial pressure of only 7 mm Hg, O_2 strongly inhibited the reduction of DBA^{++} to DBA°. As a control to detect possible reoxidation of DBA°, a cuvet was opened and bubbled with O_2 for 3 minutes; no significant oxidation was observed. A further control indicated that the enzyme retained full activity during this time. The inhibition by O_2 of enzymically induced luminescence of DBA^{++} thus indicates that reduction of DBA^{++} is a necessary prerequisite to the light-emitting reaction. The time-dependent increase of light intensity reported by Totter *et al.* (1960a) in unstirred reaction mixtures appears to reflect the gradual decrease of O_2 tension from initially inhibitory levels.

Enzymic Reduction of DBA^{++}.—Anaerobic incubation of xanthine and a slight molar excess of DBA^{++} with xanthine oxidase resulted in reduction of one mole of DBA^{++} per mole of xanthine. However, when xanthine was present in greater than two-fold molar excess over DBA^{++}, two moles of xanthine were oxidized per mole of DBA^{++} reduced, provided sufficient time was allowed to complete the reaction. These results indicate that DBA^{++} is readily and preferentially reduced to DBA° by the enzyme, and that DBA° can be further reduced to H_2DBA only after the supply of DBA^{++} is exhausted.

Although no spectrophotometrically detectable

DBA° accumulated during aerobic (20% O_2 in gas phase) incubation of DBA^{++} with xanthine oxidase and xanthine, the transient formation of a reduced form of DBA^{++} under these circumstances was strongly suggested by the following facts: The reduction of cytochrome c by xanthine oxidase, in the presence of its substrates, exhibits an absolute dependence upon the presence of molecular O_2 which can be alleviated by quinones such as menadione (Fridovich and Handler, 1962). Whereas myoglobin inhibits the O_2-dependent reduction of cytochrome c by this enzyme, it is without effect on the menadione mediated process (Fridovich, 1962). Similarly, DBA^{++} was found to mediate a myoglobin-insensitive reduction of cytochrome c which was also observed in the absence of O_2. This observation strongly suggests alternate reduction and reoxidation of DBA^{++} under these circumstances, although no DBA° was detected while this system operated in a steady state.

Effects of Metal-Binding Agents.—Enzyme-induced luminescence of DBA^{++} was markedly enhanced by cyanide, bovine serum albumin, amino acids, EDTA, and other chelating agents. Representative results are shown in Table I. The action of this diverse group of reagents appears to reflect prevention of the *activity-dependent* inhibition of xanthine oxidase by trace metals, previously described (Fridovich and Handler, 1962). Thus, the postulate (Totter *et al.*, 1960b) that cyanide acts by specifically enhancing the rate of production of radicals by the enzyme is not necessary.

TABLE I

EFFECT OF VARIOUS SUBSTANCES IN STIMULATING MAXIMUM INTENSITY

Reaction system contained Na_2CO_3, 0.05 N, *p*H 10.0; hypoxanthine, 10^{-4} M; DBA^{++}, 10^{-4} M; stimulators as marked; total volume 1.0 ml. Reaction started by adding 0.16 units of xanthine oxidase.

Stimulator	Concentration	Relative Intensity
None	—	1
Bovine serum albumin	500 μg/ml	30[a]
EDTA	1×10^{-4} M	40[a]
Serum albumin + EDTA	500 μg/ml; 1×10^{-4} M	90
Tiron	5×10^{-4} M	6[b]
Tryptophan	2.5×10^{-4} M	7

[a] Maximum stimulation. [b] Acts simultaneously as inhibitor (see Table II).

Inhibition Studies.—All compounds tested for inhibition of enzyme-induced chemiluminescence were first tested for quenching in the nonenzymic H_2O_2-DBA^{++} reaction and, where necessary, appropriate corrections for observed quenching were made. In those instances in which the potential inhibitor was a metal-binding agent, the inhibitor effect was determined under conditions of maximum protection by bovine serum albumin. The results of these studies are summarized in Table II. Tiron, Tris, and myoglobin, which have been shown to inhibit the generation of oxygen ($O_2^-\cdot$) radicals by xanthine oxidase (Fridovich and Handler, 1962) are seen to inhibit enzyme-induced luminescence. Inhibition by catechol, like that caused by sulfhydryl compounds (Totter *et al.*, 1960b), probably relates to the ready reaction of these compounds with free radicals, while cytochrome c may be presumed to compete for reaction with the free radical (Fridovich and Handler, 1962).

TABLE II

INHIBITORS OF ENZYMIC CHEMILUMINESCENCE

Reaction system as in legend for Table I. For catechol inhibition, glycine buffer, 0.05 M, *p*H 10.0, was used, and EDTA, 10^{-4} M, was present.

Inhibitor	Concentration (M)	Inhibition (%)
2,4-Dinitrophenol	5×10^{-4}	60[a]
Tris	0.05	20[b]
Tiron	5×10^{-4}	80[b]
Semicarbazide	10^{-3}	0
Catechol	5×10^{-6}	62
Myoglobin	0.5 μg/ml	56[b]
Cytochrome c	2.2×10^{-5}	55[b]

[a] Quenched H_2O_2-induced light 40%. [b] Tested in presence of 500 μg/ml bovine serum albumin.

Enzymically Induced Luminescence of Luminol.—In contrast to the behavior of DBA^{++}, luminescence in the presence of luminol was not inhibited by higher levels of O_2. In this instance, luminescence again was dependent upon the presence of O_2 and both intensity and duration of the emission increased with increasing O_2 concentration over the entire concentration range tested (Fig. 2). Since no evidence for participation of luminol as an electron acceptor has been obtained, it would not be expected that O_2 should be inhibitory. The enhancement of luminol luminescence by increases in O_2 concentration beyond that necessary to insure maximal oxidation of substrate by reduction of O_2 to H_2O_2 resembles the influence of O_2 concentration on cytochrome c reduction (Fridovich and Handler, 1962). This strongly suggests that reduction of O_2 to $O_2^-\cdot$, rather than to H_2O_2, increases at increasing O_2 concentration and that it is reaction of this radical with luminol which is responsible for chemiluminescence in this system.

Chemiluminescence Induced by Other Enzymes.—Two other iron-flavoproteins are known to initiate the autoxidation of sulfite, a process ascribed to formation of $O_2^-\cdot$ radicals. These are rabbit liver aldehyde oxidase (Fridovich and Handler, 1961) and bacterial dihydroorotic dehydrogenase.[2] Rabbit liver aldehyde oxidase,

[2] V. Aleman-Aleman and P. Handler, in preparation.

prepared according to Rajagopalan *et al.* (1962), was obtained from Dr. K. V. Rajagopalan; dihydroorotic acid dehydrogenase, prepared according to Friedman and Vennesland (1960), was kindly supplied by Dr. V. Aleman-Aleman. The former was tested with N¹-methylnicotinamide as substrate, the latter with DPNH as substrate. Under conditions similar to those used with xanthine oxidase, both were effective in inducing chemiluminescence with DBA^{++}. Both enzymes were found to reduce DBA^{++}, under anaerobic conditions, in the presence of their substrates.

Moccasin venom L-amino acid oxidase, which does not initiate sulfite autoxidation (Fridovich and Handler, 1961), was found neither to induce chemiluminescence of DBA^{++} aerobically, nor to effect the reduction of DBA^{++} anaerobically. L-leucine was employed as substrate in these studies. Beef heart cytochrome oxidase, in the presence of ferrocytochrome c and O_2, failed to induce chemiluminescence with either DBA^{++} or luminol. Since an enzyme-bound oxygen radical which had previously been observed to initiate sulfite autoxidation (Fridovich and Handler, 1961) is intermediate in the reduction of O_2 by this enzyme, luminescence in the presence of luminol would have appeared possible. Nor was luminescence observed in a system containing xanthine oxidase, xanthine, myoglobin (see above), cytochrome oxidase, ferrocytochrome c, O_2, and DBA^{++}. Both uricase and horseradish peroxidase, neither of which can initiate sulfite autoxidation, failed to induce luminescence with DBA^{++} or luminol in the presence of their substrates.

Discussion

The available data indicate that the enzyme-induced chemiluminescence of DBA^{++} occurs as a result of two processes mediated by the enzyme: DBA^{++} reduction and production of oxygen radicals. Inhibition of either process resulted in inhibition of luminescence. Only those enzymes capable of both reducing DBA^{++} and generating oxygen radicals induced the chemiluminescence of DBA^{++}.

The nature of the reduced form of DBA^{++} which is active in chemiluminescence is uncertain. That the chemiluminescence of DBA^{++} involves both a reduction and an oxidation step has been evident from the earliest work (Gleu and Petsch, 1935) in which H_2O_2 was postulated to act as both a reducing and an oxidizing agent. DBA° or $DBA^{+\cdot}$, rather than H_2DBA, is considered to be involved in the chemiluminescent reactions for several reasons. Thus, unlike DBA°, H_2DBA cannot be oxidized with concomitant light production (Decker and Petsch, 1935; Grigorovsky and Simenov, 1951). DBA^{++} and DBA° are strongly fluorescent, while H_2DBA is not (Decker and Petsch, 1935). Anaerobic reduction of DBA^{++} by xanthine oxidase and xanthine leads preferentially to DBA°. Since aerobic production of DBA° in the steady state was not observed spectrophotometrically, it is suggested that either DBA° is reoxidized while yet bound to the enzyme, possibly by an $O_2^{-}\cdot$ radical, or that aerobic reduction leads to a different product, possibly a $DBA^{+\cdot}$ radical, which would be autoxidizable. In the former case, if $O_2^{-}\cdot$ were the oxidant of DBA°, inhibition of $O_2^{-}\cdot$ production by myoglobin (Fridovich and Handler, 1962; Fridovich, 1962) should permit DBA° accumulation aerobically. This was not observed. Therefore it seems entirely possible that $DBA^{+\cdot}$ may be the product of aerobic DBA^{++} reduction by the reduced enzyme.

The production of oxygen free radicals, probably $O_2^{-}\cdot$, by xanthine oxidase has been reported (Fridovich and Handler, 1958, 1961, 1962). The inhibition of chemiluminescence by Tiron, Tris, and myoglobin, compounds known to inhibit radical production by xanthine oxidase, but without effect on the reduction of the two electron acceptors (Fridovich and Handler, 1962; Fridovich, 1962), supports the postulated role of oxygen radicals.

DBA^{++} seems to act catalytically in light production and to be regenerated continuously. Indeed, DBA^{++} was quantitatively recovered after luminescence during the oxidation of a several-fold molar excess of xanthine. Since the quantum yield of enzymic luminescence is not known, however, the cyclic nature of the reaction cannot be stated unequivocally. The report by Totter *et al.* (1960a) that DBA° is accumulated during the luminescence reactions again reflects inadequate aeration of the reaction mixtures. No attempt to observe light emission after accumulation of DBA° during anaerobic incubation was made since free DBA° is water insoluble.

The suggestion that light emission is a consequence of the reaction of DBA° or $DBA^{+\cdot}$ with an oxygen radical is in accord with the ability of reducing agents such as dithionite, Fe^{++}, and the cathode of an electrolytic cell to cause luminescence in DBA^{++} solutions. These reducing reagents would be expected to reduce DBA^{++} to DBA° or $DBA^{+\cdot}$ and also to reduce oxygen to an oxygen radical. It is noteworthy that the $SO_2^{-}\cdot$ radical has been detected in O_2-free solutions of dithionite (Rinker *et al.*, 1959), and its interaction with O_2 would produce the postulated oxygen radical. The cathodic production of such radicals is supported by indirect evidence (Kolthoff and Jordan, 1952; Fridovich and Handler, 1961), and their production during the aerobic oxidation of Fe^{++} seems likely (Weiss, 1953; Fridovich and Handler, 1958).

A tentative reaction sequence embodying these principles may be visualized. Reduced DBA^{++} and $O_2^{-}\cdot$ are produced by the reduced enzyme. These may then react on the enzyme surface, producing an oxygenated derivative of DBA^{++} in an electronically excited state. This derivative could lose its excitation energy by emitting a photon,

and in returning to the ground state be reconverted to DBA^{++} itself. An oxygenated derivative of DBA^{++} is presumed to be the emitting species, since the luminescent light is blue whereas the fluorescence of DBA^{++} is green. Since available evidence indicates that enzymically generated $O_2^-\cdot$ remains bound to the enzyme surface, and since no light emission was observed when DBA^{++} reduction and $O_2^-\cdot$ production occurred on separate enzymes, the chemiluminescent reaction is believed to take place on the enzyme surface.

If light emission is a consequence of the interaction of DBA° or $DBA^{+}\cdot$ and an oxygen radical, and if the production of each of these is dependent, in turn, upon substrate concentration, then it should follow that the intensity of light emission varies, in the usual way, with the square of the substrate concentration. Hence, there appears to be no need to postulate (Totter *et al.*, 1960a) either two substrate binding sites on xanthine oxidase or an obligatory four equivalent reduction of DBA^{++}. The observation that luminescence is directly proportional to enzyme concentration is not in conflict with these considerations if the DBA° or $DBA^{+}\cdot$ and $O_2^-\cdot$ interact on the enzyme surface, at, or close to, their point of generation, rather than in free solution. Finally, it should be noted that the low concentrations of H_2O_2 generated during the aerobic action of the enzyme could account for less than $^1/_{1000}$ of the intensity of enzyme-induced luminescence. Indeed, the addition of 10^{-4} M H_2O_2 has no perceptible effect upon the time course of luminescence. Thus, the role of H_2O_2 in the enzymic luminescence appears to be negligible.

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Effect of Metrazol on Isolated Mammalian Cells. II. Inhibition of Synthesis of Cholesterol*

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Cultures of strain L mouse fibroblasts grown in the presence of a metabolic stimulant, Metrazol (pentamethylene tetrazol), had a lower sterol content than control cultures. Cells incubated in the presence of the drug with acetate-1-C^{14} incorporated one-third of the radioactivity normally incorporated by control cells. Cells grown in the presence of Metrazol but incubated in Metrazol-free media rapidly synthesized sterols of high specific activity. Rigorous analysis of the sterol fraction indicated that 78% of the radioactivity was present in cholesterol.

Cultured mammalian cells accumulate lipids in the lag phase following cessation of the logarithmic phase of growth (King *et al.*, 1959; Bensch *et al.*, 1961). This lipid accumulation decreases gradually as old lipid-laden cells are induced to multiply. The rate of cholesterol synthesis in cultured cells, whether L strain mouse fibroblasts or Ehrlich ascites cells, increases when protein synthesis is inhibited. These results suggested

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