Journal of Bioenergetics and Biomembranes, Vol. 14, No. 2, 1982

Mitochondrial Chemiluminescence Elicited by Acetaldehyde

Erin E. Boh,[1,3] William H. Baricos,[1] Carl Bernofsky,[1] and Richard H. Steele[1,2]

Received April 24, 1981

Abstract

Acetaldehyde-dependent chemiluminescence has been found to be a sensitive technique for the study of superoxide and hydrogen peroxide formation in beef heart mitochondria. The system responds to ATP and antimycin A with increased emission intensities and to ADP and rotenone with decreased intensities, indicating that the chemiluminescence reflects the energy status of the mitochondrion. These effects are based on the ability of acetaldehyde to react with superoxide and hydrogen peroxide to form metastable intermediates which decay spontaneously with the emission of light. Additionally, these intermediates can react with cyanide to give alternative products which can also decay with the emission of light, the cyanide-evokable chemiluminescence. The interaction of acetaldehyde with mitochondria is complex because acetaldehyde can serve as a hydrogen source for NADH and as an inhibitor (at high concentration) of electron transport, and appears to be a reducing agent for a heat-stable site that autoxidatively generates HOOH from $O_2^{\cdot-}$. Inasmuch as acetaldehyde is a metabolite of ethanol, this broad spectrum of reactivity may play a role in the hepatic and cardiac toxicity that is associated with alcoholism. The heat-stable site that generates HOOH from $O_2^{\cdot-}$ has been studied further and appears to contain vicinal dithiol which is primarily responsible for the cyanide-evokable chemiluminescence.

Key Words: Mitochondria; chemiluminescence; Acetaldehyde; superoxide radical anion; hydrogen peroxide.

Introduction

In this paper, we report on the interaction of acetaldehyde with beef heart mitochondria to produce strongly chemiluminescent products whose forma-

[1]Department of Biochemistry, Tulane University Medical Center, 1430 Tulane Ave, New Orleans, Louisiana 70112.

[2]To whom correspondence should be addressed.

[3]The work reported in this paper was carried out by Erin E. Boh in partial fulfillment of the requirements for the Doctor of Philosophy degree.

0145–479X/82/0400-0115$03.00/0

tion appears to reflect the disposition of reducing equivalents in the respiratory chain. The greatest intensities occur under conditions that favor the mitochondrial generation of superoxide anion, $O_2^{\cdot-}$, and H_2O_2, and this finding is consistent with the known ability of acetaldehyde to combine with $O_2^{\cdot-}$ and H_2O_2 to form chemiluminescent products.

The presence of H_2O_2 in biological sources has been known since Chance first described the catalase–H_2O_2 complex (see Chance *et al.*, (1979, for a review). Chance and Oshino (1971), by monitoring this complex spectroscopically, and Loschen *et al.* (1971), by employing the peroxidase-scopoletin assay, established the generation of H_2O_2 by rat liver mitochondria and pigeon heart mitochondria, respectively. In addition, Chance *et al.* (1973) and Boveris and Chance (1973) demonstrated that H_2O_2 formation appears to be maximal when electron carriers are reduced either through metabolic control as in state-IV respiration, or during uncoupled state-III respiration in the presence of antimycin A. This latter work (Boveris and Chance, 1973) established that the electron donor "leaking" to oxygen is a component of the "high-potential" pool of carriers (cytochromes, quinone, nonheme iron, and flavoproteins) that lie between the rotenone and antimycin-sensitive sites.

Boveris *et al.* (1972) suggested that ubisemiquinones and ubiquinol are the chief species responsible for succinate-related H_2O_2 formation. Hinkle *et al.* (1967), using beef heart submitochondrial particles, studied the formation of H_2O_2 during ATP-dependent reverse electron flow to O_2 and suggested that an autoxidizable electron carrier, located between cytochrome *b* and NADH dehydrogenase flavin, accepted the ATP-driven electron flow from cytochrome *b* and donated these electrons directly to O_2.

Our interest in mitochondrial H_2O_2 generation stems from earlier work that showed that microsomal lipid peroxidation induced by NADPH and ascorbic acid generates solvent-extractable peroxides that have the characteristic properties of 1-hydroxyalkylhydroperoxides (Shoaf and Steele, 1974). As these peroxides also form spontaneously in a reaction between aldehydes and H_2O_2 (Marklund, 1971) and are known to chemiluminesce (Shoaf and Steele, 1974), we reasoned that similar peroxides would be formed between acetaldehyde and mitochondrially generated H_2O_2, and that their presence could be monitored by chemiluminescent techniques. Acetaldehyde is an important metabolite that arises from the oxidation of ethanol (see Cederbaum *et al.*, (1974), for a review) and is known to have marked influences on mitochondrial function (Cederbaum and Rubin, 1977a, 1977b). We expected that the addition of acetaldehyde to mitochondria under conditions that favored the production of $O_2^{\cdot-}$ and H_2O_2 would lead to the formation of 1-hydroxyalkylhydroperoxides, and the evidence presented in this paper suggests that such a reaction does indeed occur. A preliminary account of this work has appeared (Boh *et al.*, 1980).

Methods and Materials

Isolation of Beef Heart Mitochondria

Beef hearts were obtained from a local abattoir, and the mitochondria were isolated by a modified procedure of Schneider (1948). The heart muscle was finely minced in 0.01 M potassium phosphate (pH 7.4), 0.25 M sucrose, and 0.5 mM EDTA and homogenized (1:7 w/v) with a glass/Teflon Potter-Elvehjem homogenizer using a Tri-R Stir-R motor run at 7200 rpm for six 20-sec intervals, with 20-sec intermediate cooling periods. The homogenized tissue was made to a final dilution of 1:10 with the above medium and centrifuged in an International, PR-2 Centrifuge at 600 × *g* for 5 min to remove nuclei and cellular debris. The supernatant layer was decanted and recentrifuged in a Beckman, Model L Ultracentrifuge using a Type 30 Rotor at 10,000 × *g* for 10 min to obtain a mitochondrial pellet. The mitochondrial pellet was washed and resuspended in 0.25 M sucrose so that 1 ml contained the mitochondria from 2 g of heart tissue. The entire isolation procedure was carried out at 0–4°C. Electron micrographs of mitochondrial isolates showed predominantly intact mitochondria with some myofibrillar contamination.

By following the recommendations of Greiff *et al.* (1961), we have preserved beef heart mitochondria for up to 3 weeks by storing them with 9% glycerol at −25°C. We found that glycerol has very little influence on mitochondrial oxygen consumption, with or without substrate. Although glycerol attenuates somewhat the acetaldehyde-dependent chemiluminescence, it does not alter the overall nature of the responses obtained. Prior to use, all mitochondrial preparations were examined for state-III respiration using the oxygen electrode, and only those preparations that demonstrated a respiratory control index of at least 2.4 with α-ketoglutarate as substrate were used.

Preparation of Submitochondrial Particles (SMP)

SMP were prepared by sonication as described by Nohl and Hegner (1978) and modified as follows. A 4-ml sample of mitochondria containing 16 mg of protein per milliliter in 0.25 M sucrose was sonicated at 40 W with a Branson Sonifier (Model J-17A) for four 15-sec bursts with 15-sec cooling intervals at 0–4°C. The sonicate was then centrifuged at 100,000 × *g* for 105 min to sediment the particles.

Unless stated otherwise, the reaction mixtures for the chemiluminescence experiments and the oxygen consumption measurements contained the following components (abbreviations used in the figures are in parentheses): reagent mixture (reg), 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 2.5 mM $MgCl_2$, 10 mM potassium phosphate (pH 7.4); succinate

(succ), 20 mM; α-ketoglutarate and malate (KG), 20 mM and 1 mM respectively. Where added, other component concentrations were: rotenone (ROT), 5 μM (added in 0.1 ml of absolute ethanol); antimycin A (ANT), 1 μg (added in 1 μl of absolute ethanol); potassium cyanide (CN^-), 1 mM; cysteine, 0.03 mM; ADP, 0.15 mM; ATP, 0.15 mM; mitochondria (MIT), given as total milligrams of mitochondrial protein in the final volume; acetaldehyde (CHO), concentration stated in each legend. Final volume, 3 ml; temperature, ambient, 22–25°C.

Chemiluminescence was measured with a Packard, Model 3320 liquid scintillation spectrometer, adjusted to maximum gain and window width and operated in the out-of-coincidence mode. Signals from the two photomultipliers were summed and used as the input to a Packard, Model 280A ratemeter, which has a linear range of up to 1×10^6 cpm and an output of 1 mV full scale. Under these conditions, empty 20-ml dark-adapted scintillation vials (abbreviated ev in the figures) give a background count of approximately 3×10^3 cpm. Signals were recorded continuously with a Packard strip-chart recorder and monitored simultaneously on a modified Monroe calculator. All experiments were performed in a darkened room under red actinic light to prevent photoactivation of the vials and their contents.

Oxygen consumption was measured using a Rank Brothers (Bottisham, Cambridge, England) platinum electrode connected to a Packard strip-chart recorder. Protein was determined by the method of Lowry *et al.* (1951) employing bovine serum albumin as the standard. Hydrogen peroxide generation was measured fluorometrically with horseradish peroxidase and scopoletin (Andreae, 1955), employing an Aminco-Bowman Spectrophotofluorometer. The reaction mixture contained: 50 mM HEPES—0.125 M KCl buffer, pH 7.4 (19), 0.25 mM $MgCl_2$, 10 mM potassium phosphate, pH 7.4, 3.2 μM scopoletin, 10 μg/ml of horseradish peroxidase (HRP), 10 mM succinate, and 0.9 mg of SMP protein in a total volume of 3 ml. HOOH generation was initiated by the addition of 1 μg of antimycin A, and the assay system calibrated by additions of an HOOH standard.

Chemicals

ADP, ATP, antimycin A, cysteine-HCl, HEPES, α-ketoglutarate, malate, *N*-ethylmaleimide, scopoletin, succinate, superoxide dismutase (EC 1.15.1), and horseradish peroxidase (EC 1.11.1.7) were obtained from the Sigma Chemical Co., Catalase (EC 1.11.1.6) was from Worthington Biochemical Corp., and 1,4-diazabicycle(2,2,2)octane (DABCO) was from Aldrich Chemical Co. Acetaldehyde was obtained from J.T. Baker Co. and Matheson-Coleman-Bell Co. The acetaldehyde was distilled and used as a 2

M solution (Nohl and Jordan, 1980), although in some cases, the pure compound was used. All other chemicals were reagent grade, and solutions were prepared with water that was deionized and distilled with a Pyrex apparatus.

Results

Figure 1 shows that the addition of a high concentration of acetaldehyde (24 mM) to beef heart mitochondria produces a biphasic chemiluminescent response. For both systems depicted, the mitochondria were respiring on endogenous substrates. Initially, there is a burst of light which decays rapidly, followed by a second progressive increase in chemiluminescence. The second phase is greatly enhanced by the addition of antimycin A (Fig. 1a), an effect that is diminished by approximately 50% by the prior addition of rotenone (Fig. 1b). The initial (first phase) bursts of chemiluminescence on the addition of acetaldehyde are similar and therefore independent of the

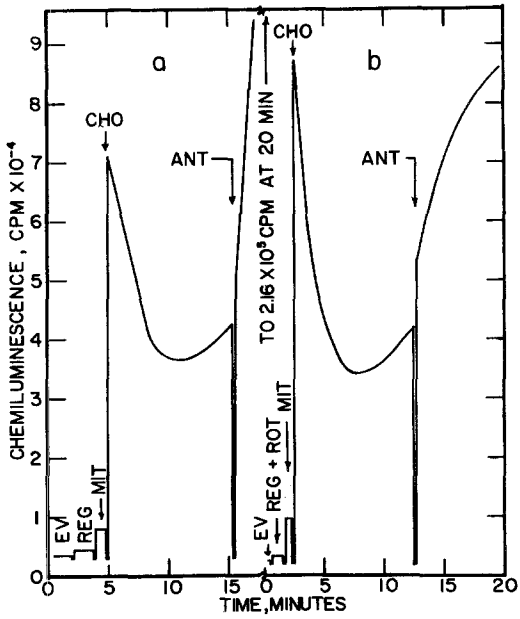

Fig. 1. Temporal traces of the mitochondrial chemiluminescence evoked by high concentrations of acetaldehyde. Systems: reg (see Methods and Materials); mitochondrial protein, 5.76 mg; acetaldehyde, 23.6 mM; curve a, no rotenone; curve b, rotenone, 5 μM added in 0.1 ml absolute ethanol; antimycin A, 1 μg, added terminally to both systems; volume, 3 ml.

presence of rotenone. These bursts probably reflect the interception by acetaldehyde of $O_2^{\cdot-}$ as it is formed from reducing equivalents that leak to oxygen. Further, the high concentrations of acetaldehyde inhibit the electron transport chain (Cederbaum and Rubin, 1977a) which may result in more $O_2^{\cdot-}$ generation. The rotenone suppression of the antimycin-enhanced chemiluminescence suggests that a site of $O_2^{\cdot-}$ formation is rotenone sensitive. In support of this contention, Cadenas *et al.* (1977) demonstrated that the rotenone-sensitive NADH-ubiquinone reductase-ubiquinol cytochrome *C* reductase is largely responsible for $O_2^{\cdot-}/H_2O_2$ formation. The bursts of chemiluminescence by high acetaldehyde, its independence of rotenone, and the observation that cyanamide, a specific inhibitor of aldehyde dehydrogenase (Cederbaum, personal communication), does not affect these bursts of chemiluminescence suggest that acetaldehyde is not the source of reducing equivalents for $O_2^{\cdot-}$ formation. This chemiluminescence depends essentially

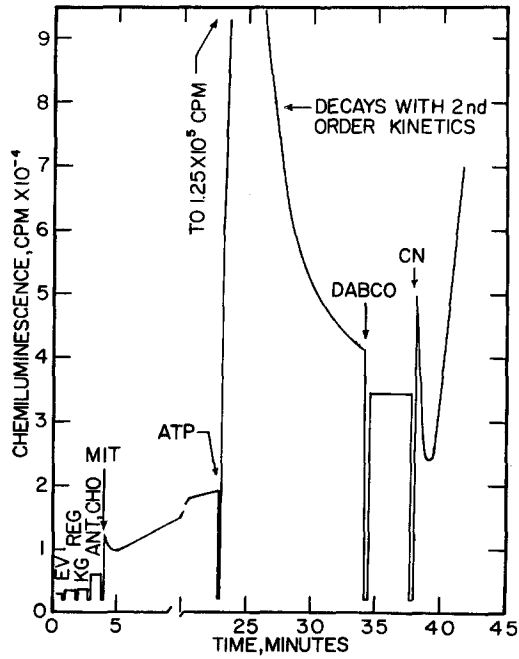

Fig. 2. Temporal trace of the acetaldehyde-dependent chemiluminescence from mitochondria respiring on α-ketoglutarate illustrating the requirement for mitochondria and the influences of antimycin A, ATP, and DABCO on the intensities. System: reg (see Methods and Materials); α-ketoglutarate, 20 mM; acetaldehyde, 5.9 mM; antimycin A, 1 μg; mitochondrial protein, 5.28 mg; ATP, 0.15 mM; DABCO, 0.03 mM; CN^-, 1 mM; volume, 3 ml.

on acetaldehyde, superoxide, hydrogen peroxide, and probably membrane-bound carbonyl.

As shown in Fig. 2, lower concentrations of acetaldehyde (6 mM) elicit considerably less chemiluminescence and only in the presence of a respiratory substrate and a respiratory inhibitor. In this experiment mitochondria were added to a reagent system containing α-ketoglutarate, antimycin A, and acetaldehyde. The gradual increase in chemiluminescence represents the gradual metabolic generation of HOOH known to be mediated by the presence of antimycin A (Boveris and Chance, 1973). However, the addition of ATP, which mediates reverse electron transport in inhibited mitochondria with the promotion of $O_2^{\cdot-}$ and HOOH production (Hinkle *et al.*, 1967), triggers a dramatic increase in the chemiluminescence which decreases with second-order kinetics. The addition of 1,4-diazabicyclo(2.2.2)octane (DABCO), a probe for singlet oxygen (Deneke and Krinsky, 1977), caused no enhancement in the emission intensity and appears to negate singlet oxygen involvement in the ATP-mediated light reaction. The terminal addition of cyanide to the system elicits a biphasic emission which we have termed a cyanide-evokable chemiluminescence but, as discussed later, appears to involve a different mechanism. This chemiluminescence is dependent on acetaldehyde, cyanide, and a heat-stable protein component capable of generating $O_2^{\cdot-}$ and HOOH. Depending on the experimental conditions, both types of chemiluminescence can occur simultaneously, and because both are dependent on HOOH, the chemiluminescence is sensitive to conditions which effect HOOH formation.

Figure 3 illustrates the enhancement of the chemiluminescence by ATP

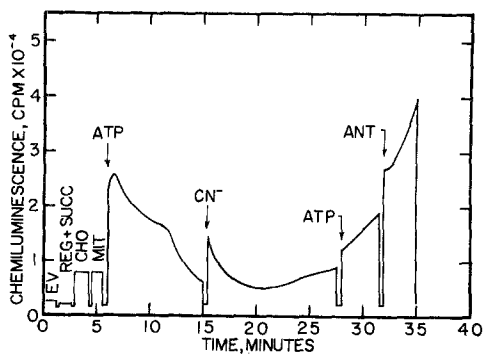

Fig. 3. Temporal trace of the acetaldehyde-dependent chemiluminescence from mitochondria respiring on succinate illustrating the differential influences of ATP and respiratory inhibitors. System: reg (see Methods and Materials); succinate, 20 mM; acetaldehyde, 5.9 mM; mitochondrial proteins, 5.28 mg; ATP, 0.15 mM; CN^-, 1 mM; volume, 3 ml.

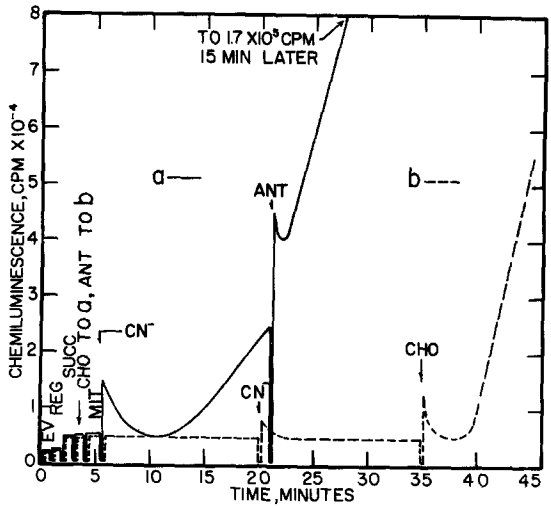

Fig. 4. Temporal traces of the acetaldehyde-dependent mitochondrial chemiluminescence illustrating the influence of respiratory inhibitors and the requirement for acetaldehyde. System: Curve a: Reg (see Methods and Materials); succinate, 20 mM; acetaldehyde, 5.9 mM; mitochondrial protein, 5.28 mg; CN^-, 1 mM; antimycin A, 1 μg, added terminally; volume, 3 ml. Curve b, same as for curve a except acetaldehyde added terminally.

when mitochondria are respiring on succinate. The intermediate addition of cyanide evokes a response which appears to be characteristic for the system respiring on succinate. A second addition of ATP elicits a second enhancement of the emission intensity which is further markedly increased by the terminal addition of antimycin A.

The mitochondrial systems shown in Figs. 4a and 4b contain the same reagents and succinate and acetaldehyde, cyanide, and antimycin A added in different order. In Fig. 4a it is evident that the addition of cyanide to the system containing acetaldehyde evokes the same biphasic succinate-type

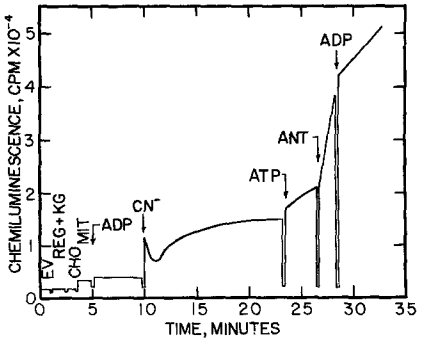

Fig. 5. Temporal trace of the acetaldehyde-dependent chemiluminescence from mitochondria respiring on α-ketoglutarate illustrating the differential influences of ADP, respiratory inhibitors, and ATP. System: reg (see Methods and Materials); α-ketoglutarate, 20 mM; acetaldehyde, 5.9 mM; mitochondrial protein, 5.28 mg; ADP, 0.15 mM; CN^-, 1 mM; ATP, 0.15 mM; antimycin A, 1 μg; volume, 3 ml.

response elicited by the intermediate addition of cyanide as shown in Fig. 3. The terminal addition of antimycin A further markedly enhanced this emission intensity. Figure 4b shows that the complete mitochondria–reagent system with both antimycin A and cyanide present displays no chemiluminescence until the acetaldehyde is added. Since the system already contained antimycin A and cyanide, the elicited emission intensity parallels the increase displayed in Fig. 4a after the addition of antimycin A. Control experiments showed that the emission depends on the presence of mitochondria and acetaldehyde.

Depicted in Fig. 5 are the temporal traces of the emissions elicited from mitochondria respiring on α-ketoglutarate, which demonstrate that ADP, reported by Boveris *et al.* (1972) to suppress HOOH formation, also suppresses the acetaldehyde-dependent emissions evoked by cyanide (compare with Fig. 2) and antimycin A.

It was not possible to demonstrate the influence of added superoxide dismutase (SOD) and catalase on the chemiluminescence from intact mitochondria. However, responses were readily obtained when submitochondrial

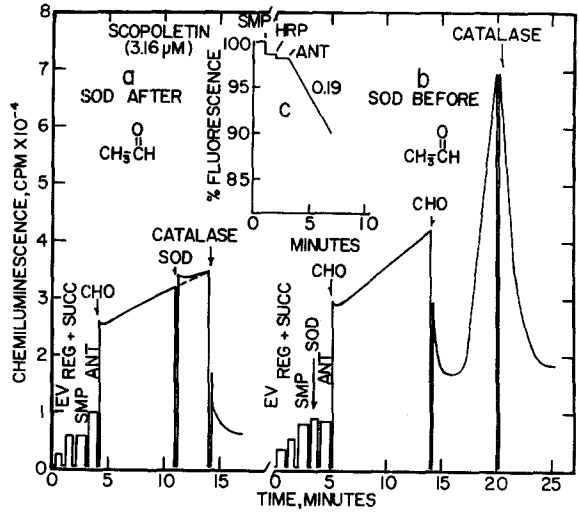

Fig. 6. Temporal traces of the acetaldehyde-dependent chemiluminescence and HOOH generation from submitochondrial particles (SMP) respiring on succinate. Systems: curves a and b illustrating the influence of SOD and catalase on the emission intensities: Reg (see Methods and Materials); succinate, 10 mM; SMP protein, 2.4 mg; antimycin A, 1 μg; acetaldehyde, 5.9 mM; SOD, 2 μM; catalase 6 nM. Curve c (inset, HOOH generation: Reg: Hepes-KCl buffer, 50 mM to 0.125 M, pH 7.4; scopoletin, 3.16 μM; horseradish peroxidase (HRP), 0.25 μM; antimycin A, 1 μg. Rate of HOOH generation: 0.19 nmol min^{-1} mg^{-1} protein. All volumes, 3 ml.

particles (SMP) were used. We present in Fig. 6, curves a and b, temporal traces for the acetaldehyde-dependent chemiluminescence from SMP respiring on succinate. Figure 6a shows that the addition of SOD to a chemiluminescing system has no influence on the emission kinetics, suggesting that the previously added acetaldehyde reacts with $O_2^{\cdot-}$ as it is formed, a reaction which has been described by Gibian *et al.* (1979). In contrast, the addition of SOD to SMP (Fig. 6b) prior to the addition of acetaldehyde produces a readily discernible accelerated chemiluminescence. The penultimate addition of cyanide to this system abruptly terminates the acetaldehyde-dependent emission and elicits the cyanide-evokable response. The terminal addition of catalase to both systems markedly suppresses the chemiluminescence and documents the importance of HOOH in both. Further proof for the production of HOOH by SMP is shown in the inset in Fig. 6c, in which the HOOH formation stimulated by the addition of antimycin A is monitored by the horseradish peroxidase mediated loss of scopoletin fluorescence.

In an effort to delineate those chemiluminescent responses which are likely to be mediated by enzyme reactions, we examined the influence of heat inactivation on the emissions. We present in Fig. 7 the temporal traces of the cyanide-evoked chemiluminescence from unheated (curve a) and from heat-treated (90°C for 10 min) (curve-b) mitochondria, both in the presence of

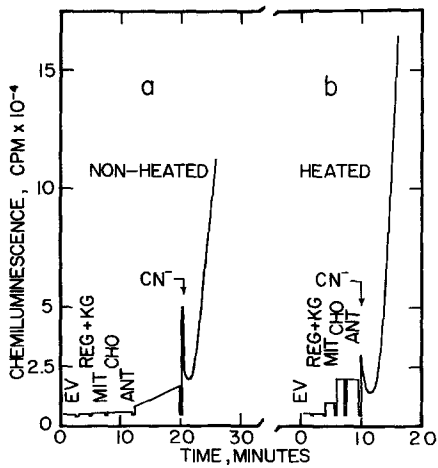

Fig. 7. Temporal traces of acetaldehyde-dependent chemiluminescence from nonheated (curve a) and heated (curve b) mitochondria in the presence of α-ketoglutarate. Systems: reg (see Methods and Materials); α-ketoglutarate, 20 mM; mitochondrial proteins, 4.9 mg; acetaldehyde, 5.9 mM; antimycin A, 1 μg; CN^-, 1 mM; volumes, 3 ml.

α-ketoglutarate. Heating abolished both the antimycin A enhanced chemiluminescence and the ADP-coupled respiration; however, there remained a slightly discernible cyanide-sensitive oxygen uptake which was independent of exogenously added succinate. Contrary to our expectations, heated mitochondria still display a cyanide-evokable chemiluminescence which requires the presence of acetaldehyde. Similar results were also obtained using heat-treated SMP. We have observed that the ability of mitochondria and SMP to display the cyanide-evokable acetaldehyde-dependent chemiluminescence depends on their ability to form formazan when heated *in the presence of nitro blue tetrazolium, NBT* (these studies will be reported in detail in a subsequent paper). Since NBT reduction requires a source of reducing equivalents resistant to temperatures which inactivate function, we considered the iron–sulfur proteins or other sulfhydryl-containing constituents as likely candidates (Palmer, 1973). We examined, therefore, the influence of *N*-ethylmaleimide (NEM), a sulfhydryl reagent (Fonyó, 1978), cysteine, and catalase on the cyanide-evokable chemiluminescence from heat-treated SMP. These results are shown in Fig. 8. Succinate was included as a reagent "substrate" in all systems. Curve a should be regarded as the control system

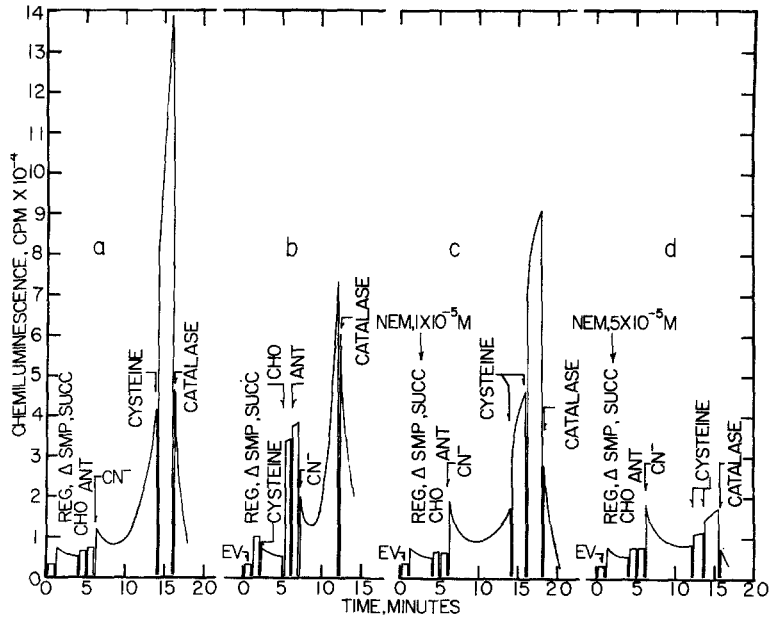

Fig. 8. Temporal traces of the acetaldehyde-dependent chemiluminescence from heated SMP in the presence of succinate illustrating the influence of cysteine, NEM, and catalase on the intensities. Systems: Reg (see Methods and Materials); SMP protein, 2.76 mg; succinate, 10 mM; acetaldehyde, 5.9 mM; antimycin A, 1 μg; CN^{-1}, 1 mM; cysteine, 30 μM; NEM: curve c, 10 μM: curve d, 50 μM; catalase, 6.6 nM.

and demonstrates the marked enhancement of the cyanide-evoked emission effected by the subsequent addition of cysteine. Curve b illustrates the influence of the prior addition of cysteine to the system on the acetaldehyde-dependent emission and on the kinetics of the cyanide-evokable response. It is evident that the prior addition of cysteine considerably enhances the intensity of the response obtained on the addition of the acetaldehyde and further markedly accelerates the kinetic response and intensities which display on the addition of cyanide. Since these results strongly implicate an involvement of sulfhydryl groups in the cyanide-evoked chemiluminescence, we examined the influence of two different concentrations of NEM on the cyanide-evoked response and its cysteine enhancement. These results are shown in Fig. 8, curves c and d, and should be compared with curve a as the control system without added NEM. It is apparent that prior addition of NEM, 10 μM, markedly attenuates the cyanide-evoked chemiluminescence and its cysteine enhancement (curve c) and results in an almost complete suppression at the 50 μM concentration (curve d). Second additions of cysteine were made to the systems depicted in curves c and d to evaluate the extent of compromised sulfhydryl-trapping effectiveness of the NEM due to the added cysteine. It is apparent in curve c that at the low concentration of NEM, a second addition of cysteine enhances the emission intensity further. We interpret this response as due to disulfide or sulfhydryl moieties which are still available for sulfhydryl exchange due either to a too low concentration of NEM, to partial removal of the NEM by the added cysteine, or, more probably a combination of both. This interpretation is supported by the data reported in curve d where the final concentration of NEM is now greater than the cysteine, 60 μM NEM. This latter result indicates that all the exchangeable sulfur moieties have been alkylated by the NEM and results in a complete suppression of the light. The terminal condition of catalase to all systems effectively suppresses the emissions.

The suppression of the cyanide-evoked and cysteine-enhanced acetaldehyde-dependent emissions from heated SMP by catalase implies that HOOH must be formed by nonenzymatic autoxidative reactions involving sulfhydryl groups. We have measured a low-level succinate-induced oxygen uptake from heated SMP at a rate of 1.6 natoms/min/mg protein. The reagent system plus cysteine, 30 μM, consumes oxygen at a rate of 29.6 natoms per minute. When the cysteine is added to heated SMP, the rate of oxygen consumption is 3.4 natoms/min/mg protein. These studies suggest an interaction between the heated SMP and the added sulfhydryl in the light reaction.

The catalase suppression of the cyanide-evoked emissions and the cysteine enhancements from viable and heated SMP document the requirement for HOOH in the light reaction. If the HOOH is formed during the autoxidation of sulfhydryl groups in the heated SMP systems, the removal of

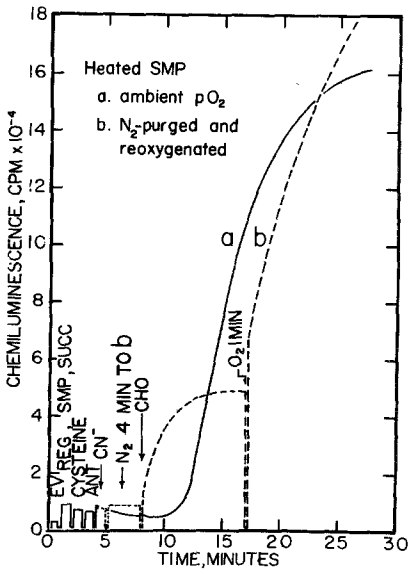

Fig. 9. Temporal traces of the acetaldehyde-dependent chemiluminescence elicited from heated SMP in the presence of succinate and illustrating the influence of low pO_2 on the intensities. Systems: reg (see Methods and Materials); succinate, 10 mM; cysteine, 30 μM; SMP protein, 2.76 mg; acetaldehyde, 5.9 mM; CN^-, 1 mM; antimycin A, 1 μg.

oxygen should attenuate the light emission. This is shown in Fig. 9. Curve a depicts the heated SMP system at ambient pO_2. N_2 purging has a marked influence on the emission characteristics. The lag phase after cyanide addition (see curve a) is eliminated, and the system quickly reaches a steady-state level, markedly less than the system at ambient pO_2. Reoxygenation restores the chemiluminescence to levels obtained at ambient pO_2. This suggests that O_2 is required for the chemiluminescence. The abrupt enhancement of the chemiluminescent intensity on acetaldehyde addition observed with N_2-purged heated SMP (Fig. 9, curve b) compared with the lag phrase shown in curve a for the system at ambient pO_2 may represent carbonyl triplet emission which can display at low pO_2 but which would be quenched at higher pO_2 (Kahn and Kasha, 1976).

Discussion

This work shows that the conditions which lead to an enhancement of chemiluminescence from mitochondria are similar to those known to cause the generation of HOOH. The extensive works of Chance *et al.* (Chance *et al.*, 1979; Chance and Ohsino, 1971; Loschen *et al.*, 1971; Chance *et al.*, 1973; Boveris and Chance, 1973; Boveris *et al.*, 1972; Hinkle *et al.*, 1967) have shown that antimycin A and cyanide stimulate HOOH generation in mitochondria and that ATP enhances its rate of formation. ADP, which

induces State-III respiration (Boveris *et al.*, 1972), has been shown to suppress HOOH formation. In our studies, ADP suppresses, and ATP and antimycin A enhance, the mitochondrial chemiluminescence. Further evidence for HOOH involvement is the enhancement of the chemiluminescence by SOD and its marked suppression by catalase.

The chemiluminescent reaction mechanisms are multifaceted and complex and may be conveniently considered as two sets of reactions. The first set of reactions, shown in Fig. 10, requires acetaldehyde and $O_2^{\cdot-}$ (scheme a) or acetaldehyde and HOOH (scheme b). There are at least two observations in our data which we interpret tentatively as evidence for the involvement of $O_2^{\cdot-}$ in the light reaction. First, the rotenone-insensitive first-phase burst of chemiluminescence (Fig. 1), which displays on the addition of high concentrations of acetaldehyde to mitochondria, suggests a free-radical mechanism and probably reflects the reaction of the $O_2^{\cdot-}$, generated metabolically or present endogenously as a complex, with the acetaldehyde (Gibian *et al.*, 1979) to form the hydroperoxyl radical which then disproportionates by the Russell mechanism (Russell, 1957) with light emission (Vassil'ev, 1967). Second, a similar mechanism is suggested by the bursts of light which display on the addition of ATP to mitochondria respiring on α-ketoglutarate (Fig. 2) or on succinate (Fig. 3). In these reactions ATP activates an energy-dependent reverse electron flow (Hinkle *et al.*, 1967) which "leaks" to oxygen to form the $O_2^{\cdot-}$. The Russell mechanism is presented in mechanism a of Fig. 10. This reaction proceeds with exothermicities large enough to form the product carbonyl function or oxygen in electronically

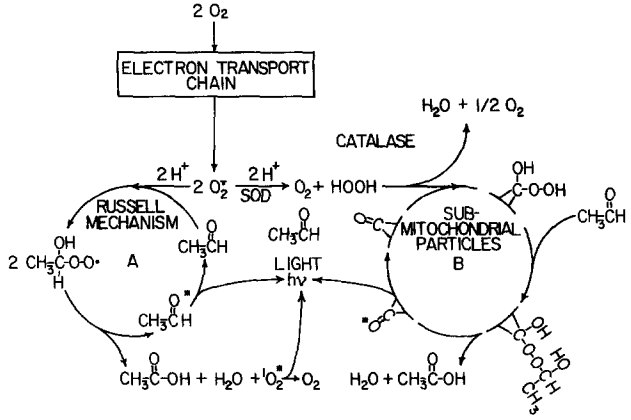

Fig. 10. Proposed mechanisms of the acetaldehyde-dependent mitochondrial chemiluminescence. Curve a depicts the $O_2^{\cdot-}$–acetaldehyde interactions resulting in chemiluminescence. Curve b depicts the HOOH–acetaldehyde interactions which result in chemiluminescence.

excited states which can relax to the ground state with light emission (Russell, 1957; Vassil'ev, 1967).

There are also at least three observations in our data which document the involvement of HOOH in the chemiluminescence reaction: first, the enhancement of the rate of emission by SOD, shown in Fig. 6, system b; second, the antimycin A induced SMP HOOH formation under conditions similar to its enhancement of the chemiluminescence (Fig. 6, system c, a, and b; see also Fig. 1, curves a and b, and Figs. 2–5); third, the suppression of the emission by catalase, shown in Figs. 6 and 8. The proposed mechanism involving HOOH in the chemiluminescent reaction is shown in Fig. 10 as mechanism b. In this mechanism we envision the HOOH formed by the disproportionation of $O_2^{\cdot-}$ as reacting with a membrane-bound carbonyl to form a hydroxyalkylhydroperoxide which is intercepted by acetaldehyde to form a bis-1-hydroxyalkylhydroperoxide. This peroxide decomposes exothermically to generate an electronically excited membrane-bound carbonyl function which relaxes with light emission. We propose this mechanism in an effort to explain the observations of kinetic and intensity data from SMP comparable with those obtained with mitochondria (compare, for example, Figs. 3 and 5 for mitochondria with Fig. 6 for SMP). Such a role for a membrane carbonyl is also consistent with the chemiluminescence elicited by the addition of HOOH to mitochondria (Steele, 1963), an observation which has been confirmed by us and reported by Boveris *et al.* (1978). A possible role for a mitochondrial carbonyl function in oxidative phosphorylation has been discussed by Lehninger (1964).

The mechanisms which we propose for the involvement of sulfhydryl groups in the chemiluminescent reactions are shown in Fig. 11, mechanism a. The demonstrated requirement of oxygen for the cyanide-evokable acetaldehyde-dependent light from heated SMP (Fig. 9), their small but finite oxygen uptake, and the suppression of the emissions by catalase (Fig. 8) suggest that HOOH is formed by the autoxidation of sulfhydryl groups. Misra (1974), employing an NBT and SOD assay, showed that the autoxidation of thiols proceeds with the formation of $O_2^{\cdot-}$ and is augmented by increasing pH. In Fig. 11 we propose that the sulfhydryls are autoxidized to form sulfur radicals and/or disulfide bonds and HOOH. We depict the sulfur radicals as catalyzing, by hydrogen abstraction, the decarbonylation of the acetaldehyde, described by Harris and Waters (1952) and Barrett and Waters (1953), and initiating a series of reactions leading to the light reaction via the Russell mechanism involving the methylperhydroxyl radical. The formation of formaldehyde on the disproportionation of the radical may explain the manner in which it is formed in the air oxidation of acetaldehyde described by Shlyapintokh *et al.* (1968). These workers, in a very thorough work, studied the chemiluminescence which accompanies the gas-phase air autoxidation of

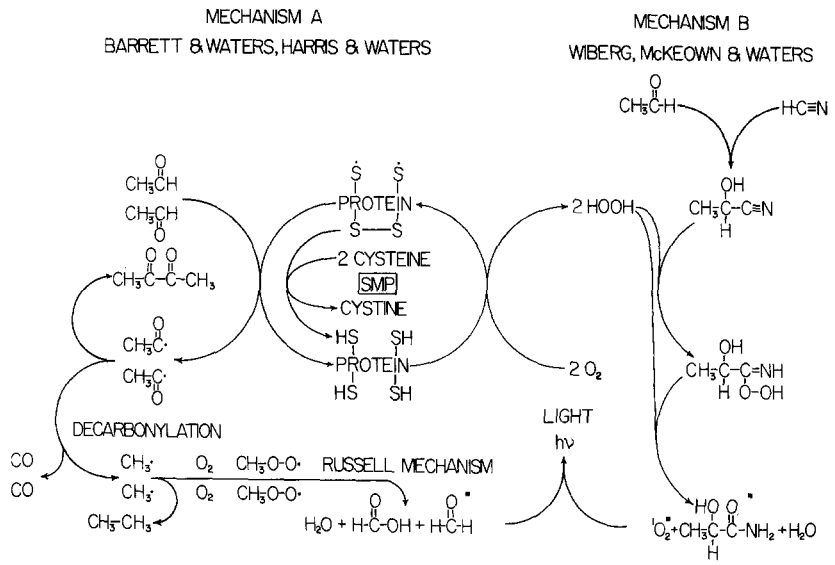

Fig. 11. Proposed mechanisms for the acetaldehyde-dependent cyanide-evoked chemiluminescence from nonheated and heated SMP. Mechanism a depicts the sulfhydryl involvement in the cyanide-evoked emissions which results in HOOH and free-radical formations. Mechanisms b depicts the cyanohydrin–HOOH reaction which can give rise to chemiluminescence.

acetaldehyde. They measured the emission spectrum which extended from 370 to 570 nm and determined that it corresponded to the fluorescence spectrum of formaldehyde which was detected in the reaction products.

The cysteine-induced enhancements of the chemiluminescence (Fig. 8, systems a and c) are probably due to the reductive exchange of the mitochondrial protein disulfide bonds (White, 1967) to regenerate vicinal sulfhydryl groups:

$$\begin{array}{lcl} \text{Protein} + \text{RSH} \rightarrow & \text{Protein} \xrightarrow{\text{RSH}} & \text{Protein} + \text{R—S—S—R} \\ \text{S——S} & \text{HS} \quad \text{S–S–R} & \text{HS} \quad \text{SH} \end{array}$$

The reactions approach completion with an excess of reducing agent and are markedly catalyzed by $O_2^{\cdot-}$ (Nagano *et al.*, 1980) in a reaction facilitated by the ability of $O_2^{\cdot-}$ to reduce disulfide bonds (Graveland *et al.*, 1980). The cysteine–disulfide exchange reaction is schematized in Fig. 11 as the inner-circle reaction of mechanism a. As depicted, chemiluminescence should display by the Russell mechanism in the absence of cyanide and without a requirement of HOOH. We suggest that these reactions explain the marked increment in the emission intensity elicited from heated SMP by the addition of cysteine before the addition of acetaldehyde and both prior to cyanide (Fig. 8, system b; compare with system a).

In mechanism b of Fig. 11, HOOH, formed by the autoxidation of sulfhydryl groups in mechanism a, reacts with the nitrile group of the cyanohydrin, known to be formed in an equilibrium reaction between hydrogen cyanide and acetaldehyde (Lapworth and Manske, 1928), to form a peroxide which on reaction with a second molecule of HOOH leads to a chemiluminescent reaction. McKeown and Waters (1964) examined this reaction, first described by Wiburg (1953), for chemiluminescence and observed both a blue light and a red light (from singlet oxygen) in the reaction of HOOH with benzylcyanide and other nitriles.

There are results in our data which have conceptual significance concerning the interaction of reducing equivalents from substrates with the disulfide–sulfhydryl moieties. The cyanide-evokable acetaldehyde-dependent emissions, which appear to be sulfhydryl-mediated, display with biphasic kinetics and intensities which reflect the substrate type, α-ketoglutarate or succinate. Furthermore, the substrate-characteristic response displays whether the cyanide is added at the beginning (Fig. 4, succinate), the middle (Fig. 3, succinate; Fig. 5, α-ketoglutarate), or terminally (Fig. 2, α-ketoglutarate) within a given sequence of component additions. The biphasic response is more rapid and intense when α-ketoglutarate is the substrate than when succinate is the substrate. Since the cyanide-evokable emissions are dependent on sulfhydryl groups, the more rapid kinetic display and intensity implies a higher concentration of sulfhydryls in the systems employing α-ketoglutarate than in the systems employing succinate, i.e., the kinetics and intensities reflect the concentration of available sulfhydryl moieties. This thesis is supported by the data of Fig. 8 where the available sulfhydryl groups were increased by the addition of cysteine, or depleted progressively by the additions of NEM. The data shown were obtained from heated SMP, and system a should be viewed as the control. The prior addition of cysteine (system b) affords a cyanide-evokable emission similar to the α-ketoglutarate response and reflects an increased availability of autoxidizable sulfhydryl groups. As the available sulfhydryl groups are progressively depleted by increasing concentrations of NEM (systems c and d), the cyanide-evoked emissions begin to resemble the succinate response. These emissions appear to reflect the redox status of disulfide–sulfhydryl moieties within the mitochondrial redox chain which are contiguous with the substrates.

There are several reports on chemiluminescence which may be relevant to our studies. Rudi and Wampler (1979) reported a low-level chemiluminescence from acidic DMSO solutions of copper(I) chloride, HOOH, and aldehydes, including acetaldehyde. They suggested that the active intermediates in the light reaction were 1-hydroxyalkylhydroperoxides. Shoaf and Steele (1974) reported a nucleophile (CN^-) elicited chemiluminescence from 1-hydroxyformylhydroperoxide. Arneson (1970) reported a HOOH-

mediated chemiluminescence during the oxidation of acetaldehyde or xanthine by milk xanthine oxidase which is known to produce $O_2^{\cdot-}$ in this reaction. DiLuzio and Stege (1977) described an acetaldehyde-enhanced chemiluminescence from rat liver homogenates which they attributed to acetaldehyde-facilitated lipid peroxidation. Stauff and Ostrowski (1967) observed a fluorescer-dye enhanced chemiluminescence from mitochondria which appeared to involve $O_2^{\cdot-}$ and singlet oxygen. Boveris *et al.* (1978) recently reported a chemiluminescence from mitochondria evoked by organic peroxides. The emission displays kinetics and intensities markedly different from those reported in our work.

This work promises to provide novel, highly sensitive, readily accessible, noninvasive methods for the temporal monitoring, by chemiluminescence, of the intramitochondrial generation and disposition of $O_2^{\cdot-}$ and HOOH. The cyanide-evokable emissions appear to reflect the redox status of membrane-bound disulfide–sulfhydryl moieties which are contiguous with the redox chain. These studies may provide new insights into the mechanisms underlying the pervasive toxicity and pathology of alcoholism.

Acknowledgments

This work was supported by the "Lucita Ward—American Heart Association—Louisiana, Inc. Award" to RHS and WHB, and in part by NIH Bio. Med. Res. Support Grant numbers 2-SO7-RR05377-16 to WHB and 2-SO7-RR05377-19 to RHS; we also acknowledge a grant from the Ethyl Corporation toWHB and RHS, and grants NSF (PCM-7722793) and NIH (HL-24192) to CB. We are also grateful to Dr. P. Kadowitz for providing bovine heart tissue, NIH, HL 18070.

References

Andreae, W. A. (1955). *Nature* **175,** 859–860.
Arneson, R. M. (1970). *Arch. Biochem. Biophys.* **136,** 352–360.
Barrett, K. E. J., and Waters, E. F. P. (1953). *Trans. Faraday Soc.* **14,** 221–227.
Boh, E. E., Baricos, W. H., Bernofsky, C., and Steele, R. H. (1980). *Fed. Proc.* **39,** 2058 (*Abs*).
Boveris, A., and Chance, B. (1973). *Biochem. J.* **134,** 707–716.
Boveris, A., Oshino, N., and Chance, B. (1972). *Biochem. J.* **128,** 617–630.
Boveris, A., Chance, B., Filipkowski, M., Nakase, Y., and Paul, K. (1978). In *Frontiers of Biological Energetics,* Vol. 2, Academic Press, New York, pp. 975–983.
Cadenas, E., Boveris, A., Ragan, C., and Stoppani, A. (1977). *Arch. Biophys. Biochem.* **180,** 248–257.
Cederbaum, A. I., and Rubin, E. (1977a). *Arch. Biochem. Biophys.* **179,** 46–66.
Cederbaum, A. I., and Rubin, E. (1977b) *Biochem. Pharmacol.* **26,** 1349–1353.
Cederbaum, A. I., Lieber, C. S., and Rubin, E. (1974). *Arch. Biochem. Biophys.* **161,** 26–39.
Chance, B., and Oshino, N. (1971). *Biochem. J.* **122,** 225–233.
Chance, B., Boveris, A., Oshino, D., and Loschen, G. (1973). In *Oxidases and Related Redox Systems,* Vol. 1, University Park Press, Baltimore, pp. 350–353.

Chance, B., Sies, H., and Boveris, A. (1979). *Physiol. Rev.* **59,** 527–605.
Deneke, C. F., and Krinsky, N. I. (1977). *Photochem. Photobiol.* **25,** 299–304.
DiLuzio, N. R., and Stege, T. E. (1977). In *Alcohol and the Liver* (Fisher, M. M., and Rankin, J. G., eds.), Plenum Press, New York.
Fonyó, A. (1978). *J. Bioenerg. Biomembr.* **10,** 171–194.
Gibian, M. J., Sawyer, D. T., Ungermann, T., Tanapoonholrivat, R., and Morrison, N. M. (1979). *J. Am. Chem. Soc.* **101,** 640–644.
Graveland, A., Bosveld, P., Lichtendonk, W. J., and Moonen, J. H. E. (1980). *Biochem. Biophys. Res. Commun.* **93,** 1189–1195.
Greiff, D., Myers, M., and Privitera, C. A. (1961). *Biochim. Biophys. Acta* **50,** 233–242.
Hinkle, P. C., Butow, R. H., Racker, E., and Chance, B. J. (1967). *J. Biol. Chem.* **242,** 5169–5173.
Harris, E. F. P., and Waters, W. A. (1952). *Nature* **170,** 212–213.
Kahn, A. U., and Kasha, M. (1976). *J. Am. Chem. Soc.* **88,** 1574–1576.
Lapworth, A., and Manske, R. H. F. (1928). *J. Chem. Soc.,* 2533.
Lehninger, A. (1964). In *The Mitochondrion,* W. A. Benjamin, New York, p. 123.
Loschen, G., Flohe, L., and Chance, B. (1971). *Biochem. J.* **122,** 225–233.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265–275.
McKeown, E., and Waters, W. A. (1964). *Nature* **203,** 1063.
Marklund, S. (1971). *Acta Chem. Scand.* **25,** 3517–3531.
Misra, H. P. J. (1974). *J. Biol. Chem.* **249,** 2151–2155.
Nagano, T., Arakane, K., and Hirobe, M. (1980). *Tetrahedron Lett.* **21,** 5021–5024.
Nohl, H., and Hegner, D. (1978). *FEBS Lett.* **89,** 126–130.
Nohl, H., and Jordan, W. (1980). *Eur. J. Biochem.* **111,** 203–210.
Palmer, G. (1973). In *Iron–Sulfur Proteins,* Vol. II (Lovenberg, W., ed.), Academic Press, New York, p. 306.
Rudie, N. G., and Wampler, J. E. (1979). *Photochem. Photobiol.* **29,** 171–174.
Russell, G. A. (1957). *J. Am. Chem. Soc.* **79,** 3871–3877.
Schneider, W. (1948). *J. Biol. Chem.* **176,** 259–266.
Shlyapintokh, V. Ya., Karpukhin, O. N., Postnikov, L. M., Tsepalov, V. F., Vichutinskii, A. A., and Zakharov, I. V. (1968). In *Chemiluminescence Techniques in Chemical Reactions,* Consultants Bureau, New York, pp. 182–222.
Shoaf, A. R., and Steele, R. H. (1974). *Biochem. Biophys. Res. Commun.* **61,** 1363–1371.
Stauff, J., and Ostrowski, J. (1967). *Z. Naturforsch. Teil B* **22,** 734–740.
Steele, R. H. (1963). *Biochemistry* **2,** 529–536.
Vassil'ev, R. F. (1967). In *Prog. React. Kinet.* **4,** 307–352.
White, F. H. (1967). Methods Enzymol., **11,** 481–484.
Wiberg, K. B. (1953). *J. Am. Chem. Soc.* **75,** 3961–3964.