A CHEMILUMINESCENCE (CL) OF PHENAZINE METHOSULFATE (PMS) IN THE PRESENCE OF HYDROGEN PEROXIDE (HOOH) INDUCED BY REDUCTANTS INCLUDING REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE (NADH) AND ASCORBIC ACID (AA)*

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In a recent paper Steele (1963a) alluded to a newly discovered chemiluminescence involving the redox pigment phenazine methosulfate (PMS). When reductants including NADH and AA were added to PMS in the presence of HOOH a chemiluminescence (CL) was observed instrumentally. We feel that the following features of these systems warrant our presentation of some preliminary results at this time: a) the importance which PMS has attained as a redox carrier in biochemical studies (see, e.g., Dickens, 1936; Geller and Gregory, 1956; Singer and Kearney, 1957); b) a chemiluminescence in free solution, non-enzymatic, and "coupled" to known biochemical redox compounds under physiological conditions; c) the striking resemblance kinetically, with smaller rate constants, to CL flashes obtained using bioluminescence enzyme preparations (see Chance et al., 1940, and Schoepfle, 1940, 1941); and d) the study of such "simple" systems may provide further insights into mechanisms leading to an eventual understanding of CL (bioluminescent) phenomena.

Materials and Methods. PMS and NADH were Sigma grade. AA was obtained from Merck. HOOH was Baker's AR, 30%. CL emissions were measured as described by Steele (1963a). Absorption spectra were measured with a Perkin-Elmer 4000A spectrophotometer.

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Results. We have "solved" our data for the equations which describe the CL "flashes" (see Steele and Breckinridge, 1963). The emissions obey series first order kinetics which, at this time, we can only write cryptically as

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C .$$

Fig. 1 shows a typical CL response induced by the injection of NADH into the PMS-HOOH system. First- and second-order plots of the CL intensity show that CL decay follows first order kinetics for over 50% of the intensity drop and then reverts to second order kinetics presumably when some reactant (unidentified) is no longer in excess. This double kinetic behavior reveals effectively the actual pseudo first order character of the k_2 rate constant. Fig. 2 shows the CL response induced by the injection of AA into the PMS-HOOH system. The CL response is more prolonged in this system and the pseudo first order character of k_2 was not apparent. We give below the rate constants for the series first order equations which describe the emissions for these systems:

	k_1	k_2
NADH-PMS-HOOH system	0.532 sec.$^{-1}$	0.071 sec.$^{-1}$
AA-PMS-HOOH system	0.044 "	0.009 "

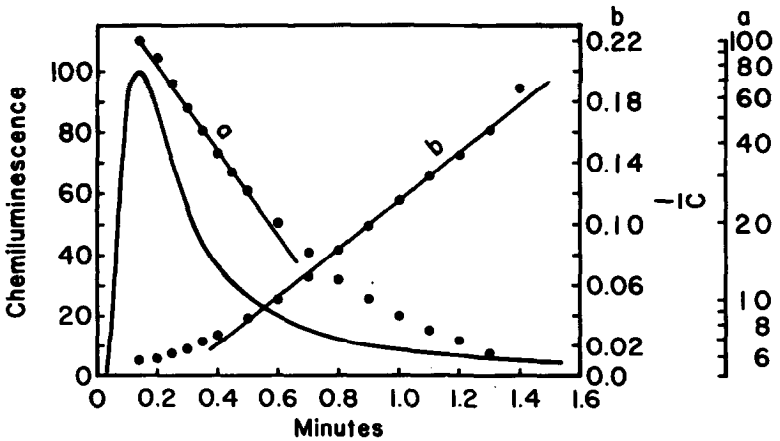

Fig. 1. Chemiluminescence vs time for a PMS-HOOH system induced by NADH addition. Decay kinetics shown: a) first order plot; b) second order plot. Final concentrations: PMS, 10^{-4} M; HOOH, 0.017 M; PO_4-buffer, 0.05 M, pH, 5.62; NADH, 1.3×10^{-4} M (1.5 mg. NADH, in 0.3 ml. H_2O, injected at zero time. Final volume, 15 ml. Temp., 25° C.

We are presently of the opinion that k_1 and k_2 for both of these systems are pseudo first order rate constants. It appears that the pseudo first order character of k_2 for the NADH-PMS-HOOH system displays itself due to the large k_1 "dumping", as it were, the reactants upon k_2 so rapidly that one reactant quickly became rate limiting. The lower value of k_1 in the AA-PMS-HOOH system (Fig. 2) prevented this overloading of k_2 and kept reactants flowing at a rate slow enough to prevent either species from becoming rate limiting. The series first order kinetics which we have found for these systems have also been observed by Steele (1963b) for the photo-induced chemiluminescence of riboflavin in water containing HOOH.

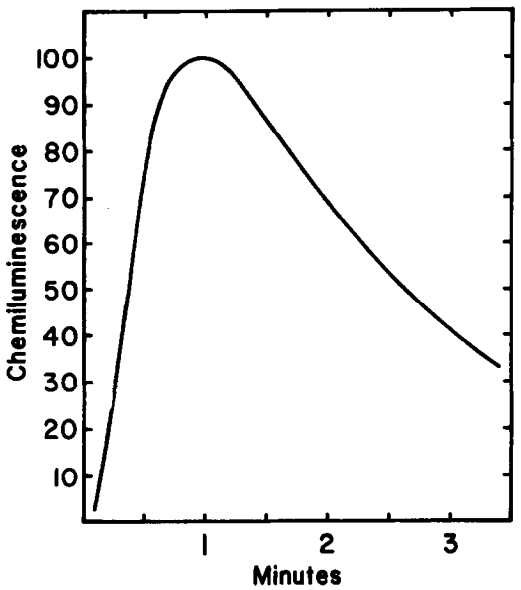

Fig. 2. Chemiluminescence vs time for a PMS-HOOH system induced by AA addition. Final concentrations: PMS, 10^{-4} M; HOOH, 0.03 M; PO_4-buffer, 0.1 M, pH, 5.53; AA, 3×10^{-4} M (0.9 ml. of 5×10^{-3} M AA was injected at zero time). Final volume, 15 ml. Temp., 25° C.

Of possible interpretative significance for the mechanisms of CL for the PMS-HOOH systems, the riboflavin-HOOH system (Steele, 1963a), and bioluminescent systems (Strehler, 1955) is what we believe may be suggestive evidence for the participation of a semiquinone (SQ) intermediate in the reactions

leading to (but not participating necessarily directly therein) light emission. When NADH is added to PMS anaerobically there appears transiently the characteristic green colored intermediate of the PMS-SQ (Swan and Felton, 1957) as the PMS is reduced to its leuco form. When oxygen is admitted to the system the leuco PMS is oxidized again transiently through the green SQ intermediate. These cyclic redox changes are analogous to those reported by Beinert (1956) for the reduction and oxidation of riboflavin and its derivatives. These results for the NADH-PMS system are presented in Fig. 3. In contrast to the reduction of PMS to its leuco form by NADH, its anaerobic reduction by AA, due essentially to the almost complete overlap of the AA and PMS potentials (AA, 0.058 v; PMS, 0.080 v, Clark, 1960), and a proper choice of concentrations, leads to an incomplete reduction of PMS to the leuco form so that the intermediate SQ is stabilized. These findings are recorded in Fig. 4 in

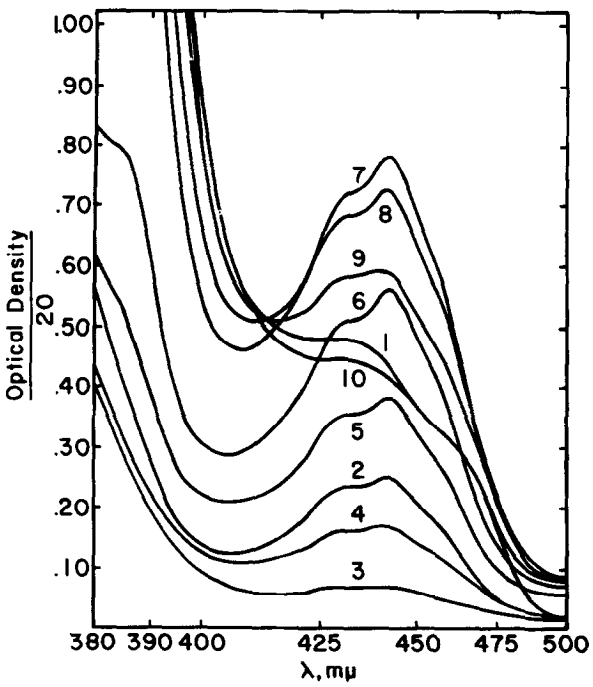

Fig. 3. Absorption spectra for cyclic redox changes via the semiquinone (SQ) for PMS induced by reduction (with NADH) and oxidation (with O_2). Systems made anaerobic initially be degassing with N_2. Curve-1, PMS; curves 2-3, show reduction, via the SQ, of PMS following 2 mg. and 1 mg. additions, respectively, of NADH; curves 4-10, changes upon subsequent oxidation by O_2 additions. Final Concentrations: PMS, 3.4×10^{-5} M; PO_4-buffer, 0.1 M, pH, 5.2; total NADH added, 1.1×10^{-4} M. Path length 10 cm. Temp., 25° C.

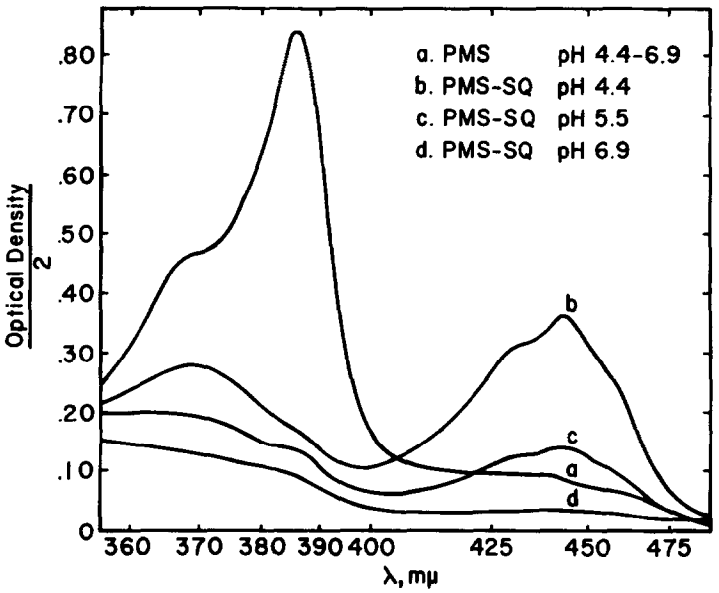

Fig. 4. PMS-SQ spectra induced by AA additions anaerobically, at different pH's in Thunberg absorption cells of 1 cm. path. Final concentrations: PMS, 6.9 x 10^{-5} M; pH's indicated on the figure. Systems: a) PMS spectrum at all pH's used; b) PMS + 0.1 M PO_4-buffer, AA, ?M (excess); c and d) PMS + 0.21 M PO_4-buffer, AA, 3.72 x 10^{-3} M. All volumes, 3.5 ml. AA "tipped" in from the side arm after 3 min. of oil pump evacuation.

which we illustrate also the known pH dependent stability of the PMS-SQ (Michaelis and Hill, 1933). Low pH stabilizes the PMS-SQ cation. It is pertinent for the concepts of the mechanisms of CL that we have never observed CL from the SQ systems in the absence of HOOH. Further, we have no direct evidence that the SQ per se is a requisite intermediate. It is our present conviction, however, that the SQ reacts either with HOOH to form a reactive species with a dissociable hydrogen (to account for the pH dependency which we have found for k_2) or that the SQ free radical acts as a reductant, in a manner analogous to ferrous iron in Fenton's reagent, with HOOH to generate OH free radicals which then react with PMS or PMS-SQ to form the molecular species which interact with light emission. Parenthetically, we should point out, however, that we have not been able to detect (by absorption spectrophotometry) the SQ in the riboflavin-HOOH system in which CL has been photoinduced (Steele, 1963a) though we have been able to demonstrate the photogeneration of OH free radicals. It may have been, however, that the steady

state concentrations of SQ, under the conditions of the experiment, were too low to measure.

Studies in progress are directed towards clarifying an observed O_2 effect on these systems (there appears to be an optimum concentration), and the use of "quenchers", e.g., halides, as an aid in identifying reactive intermediates. When we learn more about the reactions for the PMS-HOOH-reductant chemiluminescing systems we plan to resume our previously unsuccessful efforts to couple NAD-dependent enzymes to the light reaction as an assay technique.

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