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SINGLET OXYGEN FORMATION DURING PEROXIDASE CATALYZED DEGRADATION OF CARCINOGENIC N-NITROSAMINE

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Summary: The singlet oxygen traps, 2,5-diphenylfurane and 1,3-diphenylisobenzofurane were oxidized to cis-benzoylethylene and o-dibenzoylbenzene during the decomposition of diisopropyl--N-nitrosamine catalyzed by peroxidase. Singlet oxygen quenchers inhibited this conversion and also the chemiluminescence accompaying the catalyzed reaction. The chemiluminescence is enhanced by 1,4-diazobicyclo (2.2.2)octane, fluorescein, eosin rhodamine B and rose bengal but little effect was detected in the presence of 9,10-dibromoanthracene-2-sulfonate, 9,10-diphenylanthracene-2-sulfonate and anthracene-2-sulfonate. An emission spectrum of the unsensitized reaction in 560 - 600 nm region was observed. It is concluded that singlet oxygen is formed during peroxidase catalyzed degradation of diisopropyl--N-nitrosamine.

The degradation of nitrosamines by liver or kidney homogenate (1), human liver slices, plant extracts (2) and by peroxidase (3) recently has been reported. It is believed that the primary step in vivo of these oxidations is catalyzed by microsomal cytochrome P-450 mixed function oxidase (4).

From 1954 onwards many workers have investigated the biological effects of N-nitroso compounds, the majority of which are carcinogenic, mutagenic, teratogenic and can induce tumors in progeny when administered to pregnant animals (5). It was believed on the basis of earlier results that these effects are possible through alkylation of nucleic acid bases (6). However, there is no chemical reason to believe that alkylation of guanine at N-7 or O^6-alkyl should have a mutational effect, the conclusion of all more recent papers. Thus any direct causal relation be-

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tween the primary reaction and the ultimate oncogenic response appears dubious (6,7).

It was previously shown that the carcinogenic diisopropyl--N-nitrosamine (8) was decomposed by hematin and peroxidase to acetone (0.9 mol), isopropylamine (0.2 mol), molecular nitrogen, traces of nitrous oxide, and displayed chemiluminescence (3).

The importance of the excited state in biological systems was pointed out in 1965 (9) and since that time many interesting enzymic systems were studied (10,11). Evidence is presented herein for singlet oxygen formation from the reactions of nitrosamines catalyzed by peroxidase. The possible implication of singlet oxygen in the carcinogenesis of these compounds is discussed.

MATERIAL AND METHODS

Horseradish peroxidase (Type VI), catalase, SOD[1], histidine, methionine, sodium benzoate, and BHT were purchased from Sigma Chemical; 2,5-diphenylfurane from Eastman; 1,3-diphenylisobenzofurane, o-dibenzoylbenzene and DABCO from Aldrich. Eosin, riboflavine, rhodamine B, rose bengal and fluorescein were from Merck. 9,10-Dibromoanthracene-2-sulfonate, 9,10-diphenylanthracene-2--sulfonate and anthracene-2-sulfonate were prepared by the published method (11). Diisopropyl-N-nitrosamine was prepared by the method of Duran (12).

Oxygen consumption was determined with a Yellow Spring Instrument Model 53 Oxygen Monitor. The chemiluminescence and emission spectrum were measured in a Hamamatsu TV Photocounter C-767. Oxidation of DPF and DPIBF were analyzed by the method of Hawco et al. (10).

RESULTS AND DISCUSSION

Chemiluminescence was observed in the decomposition of diisopropyl-N-nitrosamine catalyzed by horseradish peroxidase at pH 7.4 (Table 1). Evidence suggesting that singlet oxygen is responsible for this chemiluminescence is inhibition by histidine, methionine, DPF and DPIBF, all known to be singlet oxygen traps (10). Also, it can be seen by the oxygen uptake that histidine and methionine did not affect markedly the formation of the intermediate. The antioxidants BHT and DTBA completely suppress oxygen uptake and hence suppress chemiluminescence by a different mechanism. No effect was observed with benzoate, catalase and SOD, which are traps for hydroxyl radical, hydrogen peroxide and

[1] Abreviations: BHT, butylated hydroxytoluene; DABCO, 1,4-diazobicyclo (2.2.2)octane; DTBA, 3,5-ditertbutyl-4-hydroxybenzylalcohol; DPIBF, 1,3-diphenylisobenzofurane; DPF, 2,5-diphenylfurane; SOD, superoxide dismutase.

TABLE I - Singlet Oxygen Formation During Peroxidase Catalyzed Diisopropyl-N-nitrosamine Decomposition

For oxygen uptake the reaction medium contained 40 mM diisopropyl-N-nitrosamine, 3 μM horseradish peroxidase (type VI) in 0.1 M phosphate buffer, pH 7.4; for chemiluminescence measurements 80 μM eosin as enhancer was added. Oxidation of 38 μM of DPIBF at 25° was followed by change in the absorption at 420 nm.

	O_2 uptake nmol/min	Integrated Photoemission at 5 min x 10^{-4} (counts)	Relative % Oxidized DPIBF (after 15 min.)
Control	720	239	100
+ 1.0 mM Histidine	520	24	83
+ 2.0 mM Histidine	360	16	63
+ 0.1 mM Methionine	620	77	81
+ 0.25 mM Methionine	540	60	71
+ 1.0 mM BHT	216	98	33
+ 2.0 mM BHT	0	0	0
+ 2.0 mM DTBA	0	0	0
+2.0 mM Benzoate	700	235	95
+ 0.1 mM DPIBF	1200	156	-
+ 0.1 mM DPF	936	150	-
+ 1.0 mM DPF	-	96	-
+ 580 units Catalase	840	210	90
+ 580 units Catalase Denatured	-	200	-
+ 100 units SOD	700	200	93
+ 100 units SOD denatured	-	215	-
Control	720	2.3[a]	100
+ 1.0 mM DABCO	730	3.2[a]	90
+ 10.0 mM DABCO	725	5.0[a]	83

a) No eosin was present

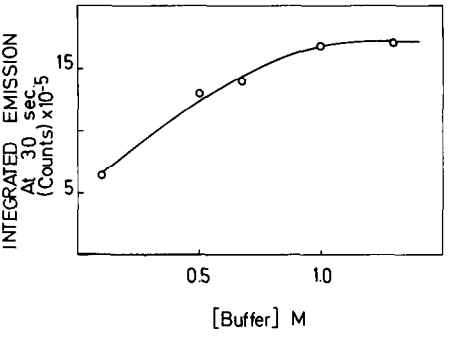


Fig. 1 - Effect of phosphate concentration on the integrated photoemission of the Diisopropyl-N-nitrosamine (8 mM)/horseradish peroxidase (2 μM)/O_2 system.

superoxide radical anion.

Other important evidence suggesting that singlet oxygen molecules are responsible for the chemiluminescence is the marked stimulation by DABCO, which enhances dimol emission in an aqueous medium (10). The only product detected in the cooxidation of DPF and DPIBF were dibenzoylethylene and dibenzoylbenzene as products of reaction with singlet oxygen.

In Fig. 1 the effect of buffer concentration on the intensity of the photoemission is shown from which the optimum value of 1.0 M phosphate buffer is deduced. The photoemission has a linear dependence on the concentration of nitrosamine over the range of 10 - 150 mM.

Table II shown the influence of different enhancers on peroxidase catalyzed diisopropyl-N-nitrosamine decomposition. The intensity of the luminescence was found to be strongly dependent on the energy of excitation, of the singlet excited states of the enhancer and not that of the triplet excited states which are very similar in all of the compounds studied. The high concentration of the dye required to achieve optimal luminescence ($\geq$0.1 mM) seems to support the mechanism of Khan and Kasha (14,15) in which a bimolecular complex of two singlet oxygen molecules is required to have sufficient energy to excite the organic substrates. The pathway by which energy pooling occurs is uncertain and a two step sucessive excitation

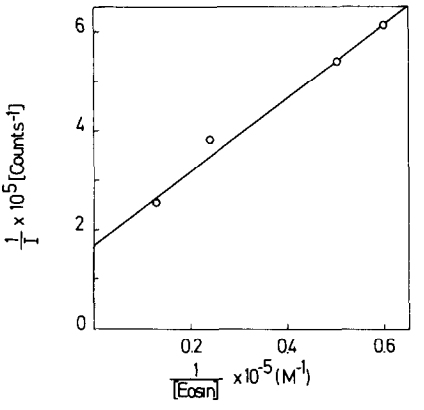


Fig. 2 - Double reciprocal plot of the effect of the eosin concentration upon the integrated emission by the system Diisopropyl-N-nitrosamine (8 mM)/horseradish peroxidase (2 μM)/O_2.

mechanism as well as a termolecular mechanism (14,15) has been proposed.

Fig. 2 shows a double reciprocal plot for eosin of the integrated photoemission and the eosin concentration (16). Using the value of K_q of 3 x 10^{10} M^{-1} sec^{-1} which is the K_q for singlet oxygen quenching by β-carotene (17) the lifetime of the excited species was 1 x 10^{-6} sec. The lifetime for $^1\Delta_g$ singlet oxygen molecule in water is 2 x 10^{-6} sec. (18). Applying the modified Perrin equation to intensities at different eosin concentrations (19) energy transfer occurs in a sphere of 160 Å radius. Probably, as suggested by Khan and Kasha (14), sensitization of the luminescence requires an actual collision between a pair of metastable singlet oxygen molecules, but energy may be transfered from the bimolecular oxygen complex to an acceptor molecule which is located some distance away. Although the emission is weak in the absence of any enhancer, an emission spectrum was observed in the region between 560 - 600 nm in the Hamamatsu TV photo-counter C-767 (20).

On the basis of product distribution, Scheme I is suggested. The α-hydroperoxy-N-nitrosamine II can decompose to zwitterion III and the latter, by collision with II by the Benson mechanism (21), can give acetone, singlet oxygen and a

TABLE II - Influence of Various Enhancers on the Integrated Photoemission During Peroxidase Catalyzed Diisopropyl-N-nitrosamine Decomposition

The reaction medium contained 8.0 mM diisopropyl--N-nitrosamine, 2 μM horseradish peroxidase (type VI) in 1.0 M phosphate buffer, pH 7.4 and 40 μM of enhancer at 40°.

Enhancer	Integrated Emission at 30 sec. counts x 10^{-4}	Excitation Wavelength nm	Flourescence[a] Emission nm	Singlet Energy Kcal/mol	Triplet[b] Energy Kcal/mol
Control	3.0	-	-	-	-
Anthracene-2-sulfonate	3.6	380	425	68	42.5
9,10-Dibromo-anthracene-2-sulfonate	4.0	403	435	66	40.2
9,10-Diphenyl anthracene-2 -sulfonate	3.5	403	440	65	40.6
Riboflavin	3.0	440	540	53	47.0
Fluorescein	42.0	500	550	52	45.2
Eosin	173.0	513	565	51	43.2
Rhodamine B	170.0	550	590	49	43.0
Rose Bengal	427.0	560	618	46	39.5

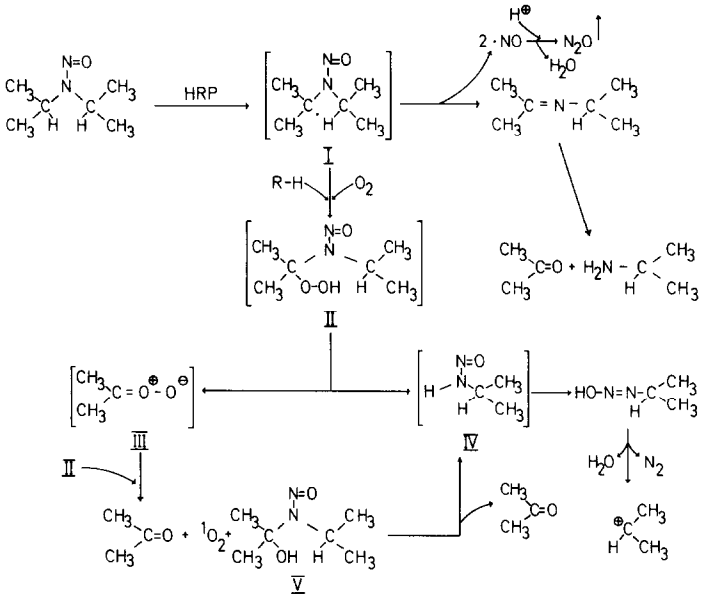
a) All the fluorescence spectra were taken at the reaction condition.

b) From ref. 13.

α-hydroxy-N-nitrosamine V.

In this paper it is shown that the singlet oxygen production could be a concomitant reaction which could play an important role in the carcinogenesis of the nitrosamines. The fact that retynyl acetate, a compound similar to β-carotene which is an excellent singlet oxygen quencher (14,22), inhibits mammary

SCHEME I



carcinogenesis induced by nitrosourea (23), and the supression of dibutylnitrosamine induced bladder carcinoma by dietary indole (24), also an excellent singlet oxygen quencher (8), might support the hypothesis that singlet oxygen plays a more important role than that of alkylation in the carcinogenic activity of the nitrosamine. Another species that could also play an important role in the nitrosamine degradation is the nitrosyl radical (·NO), the presence of which was deduced from the formation of traces of nitrous oxide (25). Recently evidence was presented of trans-nitrosation in model systems (26) and a nitroso group exchange as a way of activation of nitrosamine by bacteria (27).

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