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EXCITATION OF INDOLE ACETATE IN MYELOPEROXIDASE-H_2O_2 SYSTEM: POSSIBLE FORMATION OF INDOLE ACETATE CATION RADICAL

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Summary: Myeloperoxidase-H_2O_2-indole acetate system at pH 7.4 emitted light in visible region. Luminescent spectrum showed a weak peak at or near 480 nm and prominent peaks at or near 550, 580, and 620 nm with deep troughs near 500 and 600 nm. In some cases, no definite peak emissions near 550 and 580 nm, but a prominent broad emission between 550 and 580 nm, is observed. Such spectral patterns in the region of 510 to 620 nm were quite similar to those report for the luminescence of photo-products formed from the indole analogs (tryptophan and indole) in 50 % alcohol irradiated by U.V. (365 nm) at 77°K, assuming red shift (20-25 nm) by solvent effect. Possible formation of indole acetate cation radical (a precursor of excited indole acetate) was discussed.

In linoleate-lipoxygenase system, various indole analogs are excited in triplet states (Type I) without changes in their structures probably by the energy transfer between indole ring and an excited carbonyl produced (1). This is supported from the finding that chemiluminescent spectrum from IAA-linoleate-lipoxygenase system is quite similar to that reported for photoexcited IAA phosphorescence in EPA at 77°K, assuming red shift (35 nm)

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Abbreviations: MPO, myeloperoxidase ; IAA, indole-3-acetic acid; HRP, horse radish peroxidase.

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via a solvent effect. Such an excited IAA in the triplet state is also produced from IAA in phagocytosing leukocyte system (2). However, this system accompanies the production of other excited species which emits in the region of 550 to 620 nm. Since polymorphonuclear leukocytes contain MPO and produce H_2O_2 and $O_2^{-}\cdot$ under their activated conditions (3), spectroscopy of the light from IAA-MPO-H_2O_2 system may prove the excited species which emits the light in the region of 550 to 620 nm.

The present work reports that light in the region 510-620 nm is probably involved in an additional triplet state (Type II), which may be produced from indole acetic acid cation radical.

Materials and Methods

Enzyme preparations; Purified glucose oxidase was purchased from Boehringer Mannheim. One unit of the activity was defined as the amount of enzyme which consumed 0.1 nmole of oxygen/sec/ml of reaction mixture. The reaction mixture contained 9.3 mM glucose, 0.06 M sodium phosphate buffer (pH 7.0) and glucose oxidase in a total volume of 3 ml. Oxygen consumption was measured at 25°C in a Yanagimoto oxygenometer Model PO-100A. MPO was prepared from human polymorphonuclear leukocytes according to the methods described by Zyliczyński *et al* (4). The peroxidase activity was measured by the increase of optical dencity at 485 nm in the presence of p-phenylenediamine and H_2O_2 (5). One unit of the activity was defined as the amount of enzyme which caused an absorbance increase of 0.001/sec/3.2 ml of reaction mixture at 25°C. Specific activity, activity/mg protein, was 200. Protein was determined by phenol-biuret method (6).

Incubation; Standard incubation mixture contained 9.3 mM glucose, 5.59 units of glucose oxidase, 85 units of MPO, 200 μM IAA, and 0.06 M buffer (acetate buffer for the pH lower than 6.0 or phosphate buffer for the pH between 6.0 and 7.4) in a total volume of 3.0 ml. The reaction was initiated by the addition of glucose. In some cases, 1.1 mM H_2O_2 was added to a incubation mixture excluding H_2O_2 generating system (glucose-glucose oxidase) from the standard incubation mixture. Incubation was conducted at 25°C without shaking (except for the initiation of the reaction).

Chemiluminescent measurements; Luminescent intensity (counts/min) was measured by Luminescence Reader, Aloka BLR-101 at 25°C. Background count was approximately 35-50 counts/min. This counter is convenient for continuous detection of luminescence, i.e. counts/3 sec can be calculated to be counts/min which were recorded on the papre during incubation, even though the photomultiplyer equipped has low sensitivity, compared with that of ordinary scintillation counter. Chemiluminescent spectra were taken by our spectrometer (7).

Qualitative analysis of products; The reaction mixture was extracted 3 times with equivolume of ethylacetate containing 50 μM 2,5-di-t-butylhydroquinone, the pooled extract was dried over Na_2SO_4 and the organic solvent was removed under reduced pressure. The residue was dissolved in methanol. An aliquot of the methanol solution was then analyzed by thin layer chromatography (1) and high pressure liquid chromatography, using authentic samples as markers. The assay conditions for the latter were followes; equipment, Hitachi Model 633A; column, 3010 (1 m); eluting solvent, methanol-water (17:3 V/V); pressure, 30 kg/cm^2.

Results and Discussion

When 1.1 mM H_2O_2 was added to a reaction mixture (pH 5.0) excluding H_2O_2 generating system from the standard incubation mixture, little or no luminescence was detected by a photon counter. However, the luminescence in visible region appeard at higher pH and increased with increasing pH up to near pH 7.4, but decreased rapidly after reaching its maximum, probably because of the decomposition of H_2O_2 to O_2 by a catalase-like action of MPO (8). The luminescent reaction was greatly prolonged in the standard reaction mixture in which H_2O_2 generating system is present. Some of these results are shown in Fig. 1. To know the emitting species, the luminescence, which appeared after the peak

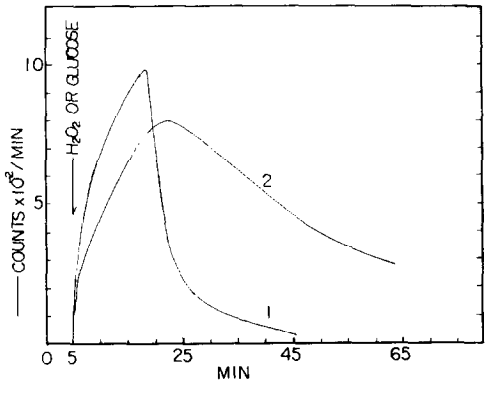

Fig. 1. Chemiluminescent intensities in the MPO-H_2O_2-IAA systems as a function of the time. The reaction was initiated by the addition of H_2O_2 for a system excluding H_2O_2 generating system or of glucose for the standard incubation mixture (arrow). The incubation was conducted at pH 7.4 and at 25°C.

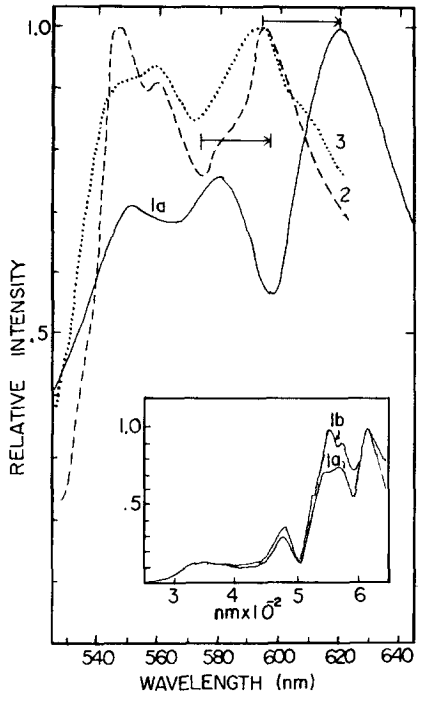

Fig. 2. Chemiluminescent spectra of MPO-H_2O_2-IAA (1a and 1b) and of the photoproduct of indole (2) or IAA (3) in 50 % ethanol at 77°K (after Vladimirov *et al.*).

emission and decaied gradually in a linear fashion, was subjected for our spectral analysis. The spectra showed a weak peak at or near 480 nm and prominent peaks at or near 550, 580 and 620 nm with deep troughs near 500 and 600 nm (Fig. 2). In some cases, no definite peak emission at or near 550 and 580 nm, but a prominent broad emission between 550 and 580 nm was observed. Such spectral patterns in the region of 510 to 620 nm are quite similar to those reported for the luminescence of photoproducts formed from indole analogs (tryptophan and indole) in 50 % ethanol irradiated by U.V. (365 nm) at 77°K (9), assuming red shift (20-25 nm) by solvent effect. Binary collision of $^1\Delta_g$ type of $^1O_2$ in aqueous system also emits in the region 500 to 640 nm; i.e. 520, 580, and 635 nm (7), but does not emit at or near 550 nm. The weak luminescence at or near 480 nm in MPO-H_2O_2-IAA is

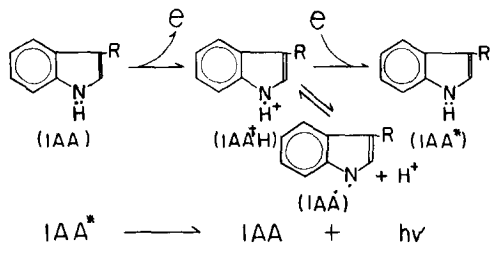

Figure 3

considered to be a phosphorescence of IAA, which has been reported from our laboratory (1), but would not be a fluorescence corresponding to $^1\Delta_g + {}^1\Sigma_g^+ \longrightarrow 2\ {}^3\Sigma_g^-$.

The luminescence in the region of 510 to 630 nm observed in the present system is considered to be involved in the reaction of IAA cation radical with solvated electron (9) or a radical anion (Fig. 3). In IAA-MPO-H_2O_2 system, abstraction of electron from NH group in indole ring could be caused by the action of MPO-H_2O_2 complex. For the excitation of indole analogs radical cation, like $IAA \cdot H^{+\cdot}$, should be remained for a while to react with solvated electron or an anion radical. However, such a radical cation can easily be deprotonated at neutral pH, judging from pK_a of 4.2 for the deprotonation of tryptophan cation radical to tryptophan neutral radical (10). On the other hand, higher pH is suitable for the stability of solvated electron or of anion radical. However, the excitation of IAA, shown in Fig. 3, could occur in protein or in enzyme with a specific nature of local environment. Electron transfer from the indole rings to the pyridine coenzymes at neutral pH has been reported by Cilento and Giusti (11). Even though the source of such reducing agents is not clarified at the present, electron may be supplied from heme-iron of MPO in the present system.

As shown in Fig. 4, the metabolites obtained from IAA during 1 hr-incubation were indole-3-carboxylic acid, indole-3-aldehyde,

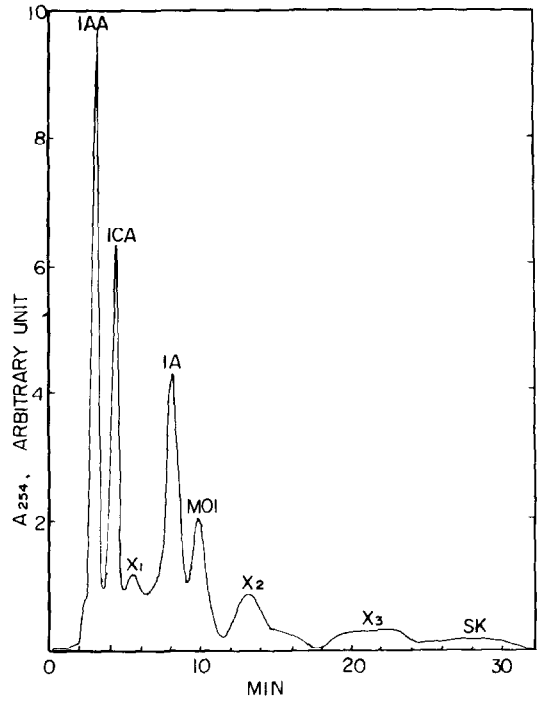

Fig. 4. Chromatographic pattern of the compounds with absorption at 254 nm (A_{254}) in the standard incubation mixture (pH 7.4) incubated for 1 hr. ICA, indole-3-carboxylic acid; IA, indole-3-aldehyde; MOI, methyleneoxyindole; SK, skatole; X_1-X_3, unknown compounds.

methyleneoxyindole, skatole, and unidentified compounds. Neither o-formylaminoacetophenone nor o-formylaminobenzoyl acetic acid ($^1O_2$ derived product) was detected by the high pressure liquid chromatography and the thin layer chromatography (1). The same metabolites could also be formed in IAA-HRP system (pH 7.4), in which chemiluminescence with main peak at or near 410 nm and shoulder at 480 nm could be observed (unpublished data). Therefore it seems unlikely that "Type II" luminescence in MPO-H_2O_2-IAA system is attended by a structual change of IAA.

Even though certain mechanism of luminescence in MPO-H_2O_2-IAA system is unknown, it is of interest that a mimic photodynamic action on IAA occurs in the dark biological system.

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