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A Study on the Mechanism of Hypericism*

Ahn, Jeong Soo
Dept. of chemistry
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〈Abstract〉

In order to establish the photochemical basis of hypericism, the following experiments were aimed at elucidating the mechanism of photosensitization by hypericin in solution: (a) photosensitizing property of hypericin, (b) solvent isotope effect on photodynamic action, (c) reduction of ferric ion by superoxide, and (d) effects of light on the activity of alkaline phosphatase in the presence of hypericin.

The photodynamic actions of hypericin involving superoxide anions, which can be produced from photoelctron transfer processes are in good agreement with these experimental results obtained.

Hypericism 현상의 메커니즘에 관한 연구*

안 정 수
화 학 과
(1980. 8. 30 접수)

〈요 약〉

Hypericism 현상의 광화학적 근거를 확립하기 위하여 hypericin의 광증감성질의 메커니즘을 밝히는 실험으로 (a) hypericin의 광증감작용, (b) 광반응의 용매동위원소효과 (c) superoxide에 의한 3가철이온의 환원, (d) enzyme의 activity에 미치는 광효과 등을 용액속에서 조사하였다.

이러한 실험에서 얻은 결과로 hypericin의 광화학작용은 광전자전달로 생성될 수 있는 superoxide anion이 관련됨을 알았다.

Ⅰ. Introduction

Hypericism is a state of skin sensitivity to visible light following ingestion of hypericin-contaning food. Hypericin(1, 3, 4, 6, 8, 13-hexa hydroxynaphthodianthrone) derives its name from Hypericum, a genus of plants which contain the natural pigments in minute glands of plant organs in various species(1). The photosensitizaton effects of skin by hypericin include skin irritation, abnormal behaviors, high body temperature, convulsions and sometimes death in animals such as sheep, cattle, horses, goats, rabbits, rats and mice, etc. The symptom disappear if the animal are kept in the dark. This disease produced by sensitization of hyericin by sunlight was termed hypericism(2). Although hypericin has been used as an antidepressant drug in man, its adverse photobiological effect on human skin and health have not been investigated.

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Following ingestion of hypericin-containing plants and other sources, the photosensitizer is usually transferred in the blood to the skin. Where skin sensitizing effects are triggered upon absorption of visible light, particularly 580~620 nm light, by the accumulated hypericin. Pace and Mackinney found that the skin sensitization in rats was not effected unless oxygen was present, thus calling the hypericism a photodynamic action(3). Pace and Blum also showed that oxygen was required for photodynamic hemolysis by hypericin(4). Furthermore, since the action spectrum of hypericism coincides with the absorption spectrum of hypericin(5), it seemed at first glance that the photobiological reactivity of hypericin in due to its photodynamic action, i.e. photosensitized oxidation of cellular components in skin analogous to many other organic dyes which are photodynamic. However, the molecular mechanism of hypericism has not been elucidated in the literature and it remains to be established.

The photodynamic action of sensitizers usually involves light-sensitized oxidation of cellular components including enzymes, membranes and nucleic acids. The photosensitization is carried out through the following sequences of photophysical and photochemical processes;

(a) Singlet oxygen mechanism (Type Ⅱ photooxidation):

The photosensitizer absorbs the light and is raised thereby from the ground state to the short-lived singlet excited state. From this state the molecule may return to the ground state or it may pass into the longerlived triplet state. The triplet state sensitizer react with ground state oxygen (in the triplet state) to form siglet oxygen, a highly reactive form of oxygen. Singlet oxygen then reacts with the substrate to produce oxidized substrate.

^1H ——→H+heat: Radiationless decay

$_1H$ →^3H: Intersystem crossing

$^3H+O_2$ ——→$H+{}^1O_2(^1\triangle_g)$: Energy transfer

$^1O_2$+substrates—›Oxidation of substrates and resulting damage to cellular systems

where left superscripts 1 and 3 stand for singlet and triplet excited states of hypericin, respectively, and $^1O_2$ designates the singlet molecular oxygen which acts as the toxic oxidizing agent in cellular systems.

(b) Electron transfer mechanism:

In photodynamic action the light energy absorbed by the sensitizer is transferred directly to form triplet-state sensitizers. The sensitizer molecule in the triplet state may react with substrate to produce a sensitizer molecule in the semioxidized state. The semioxidized substrate may then react with molecular oxygen, resulting in the oxidized state of the substrate. The semireduced sensitizer may react with molecular oxygen to form oxygen superoxide radical; the sensitizer returns to the ground state. The superoxide radical, a very potent oxidizer, may in turn oxidize the substrte(6).

H+light ——→^1H

^1H ——→H+light

^1H ——→H+heat

^1H ——→^3H

^3H+S, substrate→H$^{\cdot}$ +S$^{\cdot}$

H$^{\cdot}$ +O_2 ——→$O_2^{\cdot-}$ +H

S$^{\cdot}$ +S$^{\cdot}$ ——→S+S_{ox}

$O_2^{\cdot-}$ +cellular components—›Oxidative damages and hemolysis, etc.

where dot designates semireduced or oxidized radical species and S_{ox} represents oxidized product of substrate.

Since in photodynamic action several pathways may occur simultaneously, it is sometimes difficult to determine whicih one is predominant(6). Some 400 dfferent photosensitizers have been used in photodynamic studied with known molecular substrates than with cells.

Such studies have not been done with hypericin(1).

The approach described in this study will establish the nature of the photosensitizing activity of hypericin in terms of its ability to cause damages to cellular components via photooxidative mechanism in solution.

Ⅱ. Materials and Experimental Methods

1. Materials

Hypericin was obtained as a generous gift from Dr. P.S. Song (Texas Tech. Univ.) was purified by column chromatography on Sephadex LH-20 with Dimethylsulfoxide (DMSO: Aldrich reagent grade)(Fig. 1). Hypericin sensitized

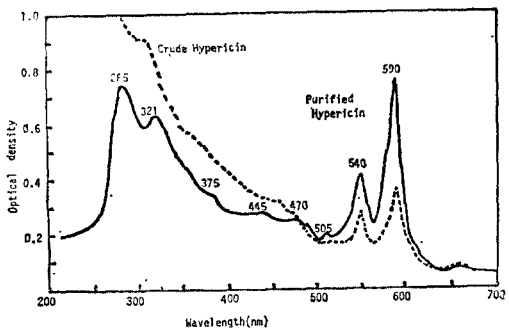

Fig. 1 Absorption Spectrum of Hypericin. Purified by Column Chromatography with Sephadex LH-20/DMSO.

reaction was done in DMSO containig about 5% H_2O. Stock solution of hypericin was irradiated for 20 min with light to convert remaining protohypericin to hypericin before use(7).

DMSO-d6 and bipyridyl were products of Sigma Chemical Corp. Akaline phosphatase and rose bengal were obtained from Aldrich and used without further purification. 1,3-diphenylisobenzofuran was kindly supplied by K.H. Yeon, KAIST, appeared to be pure by TLC-analysis in dark condition(8). All other chemicals were comercial products of the highest available quality unless otherwise stated.

2. Methods

Photooxidation of 1,3-diphenylisobenzofuran (DPBF) was sensitized in DMSO containing about 1×10^{-5}M hypericin by light from a 300W tungsten projector lamp passed through a Bausch and Lomb Monochromator (1350 grooves/mm, 300nm blaze) and a yellow cut-off filter (Corning No. 3484). During irradiation, the sample in a quartz cuvette of 1cm square section which allowed convenient concentrating in a spectrophotometer was bubbled with stream of air to maintain near O_2 saturation and to stir, and the temperature was kept at $20\pm1°C$ by means of thermostated water circulating through the cell holder. In all cases, the final concentration of sensitizer and quencher were about 1×10^{-5}M hypericin and 2×10^{-5}M DPBF. Progress of the reaction was followed by checking the fluorescence intensity of DPBF; the disappearance of DPBF as a function of the irradiation time was measured under different experimental conditions by the spectrofluorometric method. All procedures were carefully competed as soon as possible since DPBF was a sensitive compound to various oxidants. The presence of the sensitizers did not interfer with this determination. Control experiments showed that, in the range of examined concentrations, there was a linear relationship between the emission intensity and the concentration of DPBF.

Solvent isotope effect on photodynamic action by hypericin was compared in order to ascertain the role of siglet oxygen in DMSO and in DMSO and DMSO-d6.

Optical density at 540nm of the cubette contains 1.0ml each of 1.0×10^{-4}M bipyridil, 1.1×10^{-4} $FeCl_3$ and 1.1×10^{-5}M hypericin was measured under different irradiation time in order to ascertain whether Fe^{+2}-bipyridyl complex formed.

It was also tested whether or not hypericin

can generate singlet oxygen in solution by monitoring photodynamic inactivation of enzyme (alkaline phosphatase was irradiated monochromatically at 590nm while being flushed with air) whose activity can be readily assayed spectrophotometrically without significant interference by hypericin. The photodynamic inactivation of hypericin was compared with that of the enzyme by rose bengal known to be an efficient siglet oxygen generator.

UV and visible absorption spectra were measured with a Varian Techtron 635D UV-VIS Spectrophotometer. Fluorescence spectra were recorded and checked using an Aminco-Bowman Spectrophotofluorometer.

Ⅲ. Results and Discussion

1. Photosensitizing property of hypericin

From photooxidation experiments, it was found that the fluorescence intensity of the DPBF was not changed in the course of irradiation (600nm) without any sensitizer. During the reaction, the fluorescence intensity of the DPBF was checked at 450nm(excitation: 410nm, emission: 450nm). At 450nm, the fluorescence intensity of hypericin is negligible. When the photoreaction was purged of any dissolved oxygen by a current of oxygen free nitrogen, the concenrtation of DPBF was decreased very slowly during irradiation.

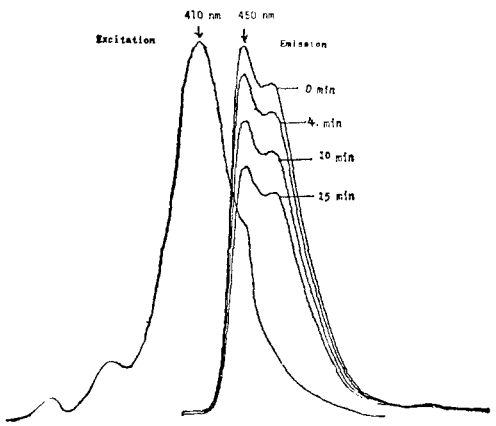

Fig. 2 Emission and Excitation Spectra of DPBF in DMSO. Hypericin (2.63×10^{-5}M) sensitized decomposition of DPBF (2.60×10^{-5}M) irradiated with wavelength of 600nm.

In Fig. 2, there is hypericin sensitized photodecomposition of DPBF. It can be seen from this figure that there is a regular change in fluorescence intensity of DPBF for the irradiation time.

Both possibilities of forming charge transfer complex and eximer of hyrericin with DPBF were eliminated by examination of their UV and fluorescence spectra before and after mixing DPBF in DMSO solution of hypericin. There was no change of absorption and emission maximum wavelength. And there was no change of optical density of hypericin in the course of photoreaction.

2. Deuterium solvent effect on photooxidation

The lifetime of singlet oxygen is known to be approximately 5 times as long in DMSO as in DMSO-d6. Therefore, about fivefold increase in the rate of photooxidation might be expected for a pure singlet oxygen reaction in going from protonated to deuterated solvent(9). However, this increase would be smaller if Type I mechanism becomes predominant or if the acceptor concentration is high enough to quench singlet oxygen significantly. In Fig. 3 the rate of hypericin sensitized photooxidation of DPBF at lower concentions of substrate shows no significant differences with that in DMSO+DMSO-d6 (33.3%) mixture. It seems probable that the hypericin sensitized photooxidation is mainly due to the contribution of nonsinglet oxygen process. However, this is no complete proof that singlet oxygen are not formed.

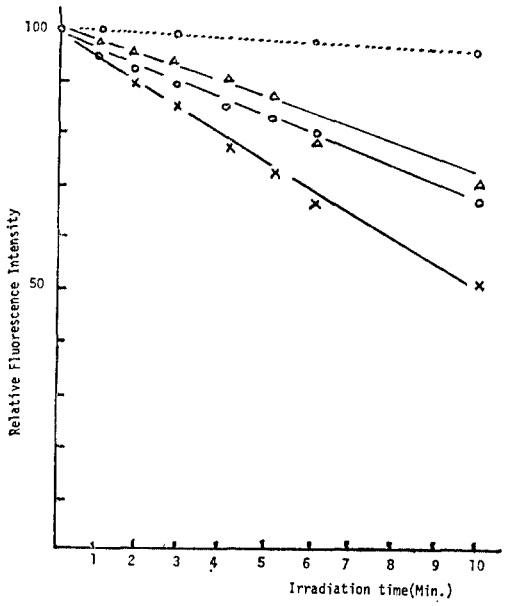

Fig. 3 Effect of Light on Fluoresence Intensity (at 450 nm) of DPBF in the Presence of Hypericin and Oxygen in DMSO, DMSO-d6 and $CHCl_3$.
—○—○—○— : in DMSO
—△—△—△— : in DMSO+DMSO-d6 (33.3%)
—×—×—×— : in $CHCl_3$
···○···○···○··· : in DMSO(absence of Hyp.)
Excitation wavelength at 410nm.

3. Reduction of Ferric ion (Fe^{+3}) by hypericin during irradiation

Fig. 4 shows that reduction of ferric ions to ferrous ions by hypericin in DMSO during irradiation is followed at 600nm. Since ferric ions are readily reduced by superoxide anion, formation of Fe^{+2}....Bipyridyl complex is caused increasing of optical density at 540nm.

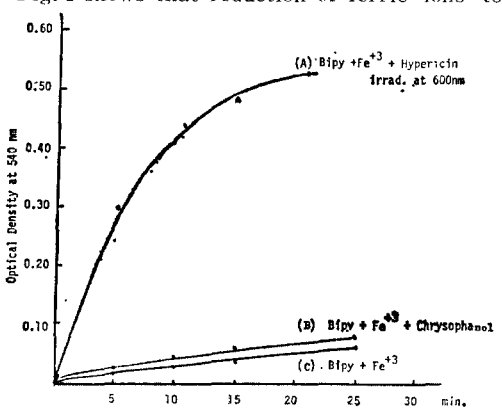

Fig. 4 Reduction of Fe^{+3} by Hypericin/DMSO. The Cuvette contains 1.0ml each of 1.0×10^{-4}M Bipyridyl, 1.1×10^{-4}M $FeCl_3$ and 1.1×10^{-5}M Hypericin(A). Incresing of OD 540 nm due to formation of Fe^{+2}-Bipyridyl complex.

4. Effect of light on the activity of alkaline phosphatase in presence of hypericin

Effects of light on the activity of alkaline phosphatase in the presence of rose bengal are shown in Fig. 5. It is well known that rose bengal sensitized photooxidations proceed exclusively via type II mechanism (singlet oxygen mechanism)(10). There is a regular decreasing in the activity of alkaline phosphatase for the irradiation time. For the effects of hypericin on enzyme activity, irradiation (wave-length at 525nm) tends to increase the activity of alkaline phosphatase. It is noted that the former results in production of singlet oxygen while the latter does another process. This

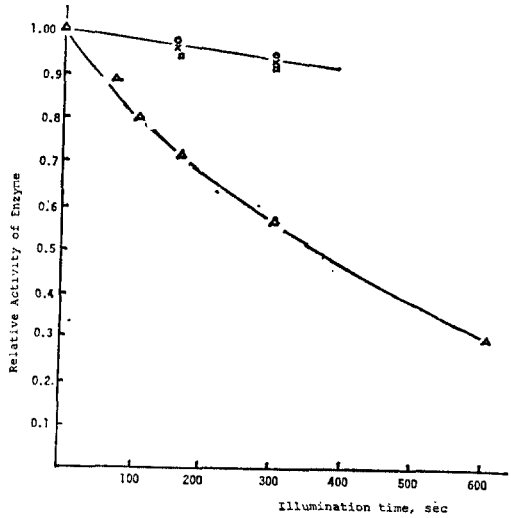

Fig. 5 Effects of Light on the Activity of Alkaline Phosphatase in the Presence of Rose bengal. Concentration of RB: 1.7×10^{-5}M, Irradiation wavelength at 525nm
—○—○— : in N_2 without light
—×—×— : in N_2 with light
—□—□— : in O_2 without light
—△—△— : in O_2 with light

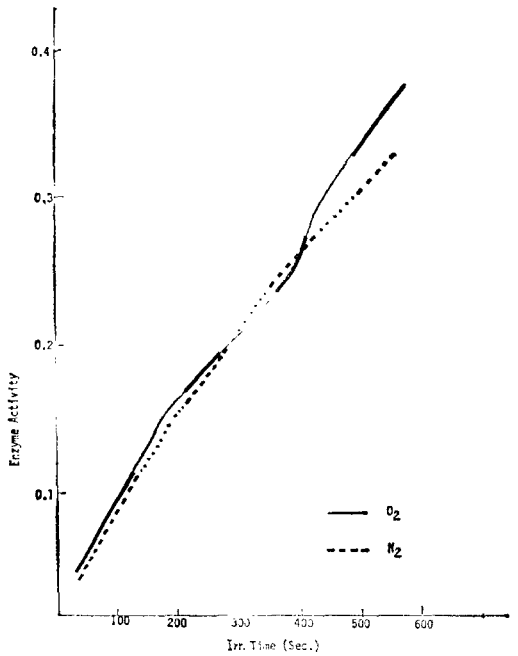

Fig. 6 Effects of Hypericin on Enzyme Activity of Bacterial Alkaline Phosphatase. Tris-HCl (pH 9.0, 1.5M) Buffer.

result may imply the importance of a nonsinglet oxygen mechanism involving interaction between enzyme and superoxide anion radical in the hypericin sensitized photoreaction.

From these results, it is concluded that hypericin is a good superoxide anion radical senitizer and hypericism is caused by photosensitization of hypericin.

Hypericism is a skindisease caused by the combined action of visible light and hypericin. Although hypericin is found in plants, it has also been synthesized as an anti-depressant drug. Since this compound is highly photodynamic and its effect in man has not been adequately investigated from the point of view of its photobiology, this study has been aimed at elucidating the molecular basis of hypericism in terms of photochemical mechanisms of the hypericin photosensitization. The present study will not only enhance our understanding of the mechanism of hypericism, but it will provide information as to its potential health hazard in man when used as drug.

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