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# Concerning the Hypericin Sensitized Photooxidation of Bilirubin IXα[a]

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**Summary.** In aerated protic solvents like ethanol or 80% aqueous ethanol, bilirubin IX$\alpha$ was found to undergo a hypericin sensitized oxidative photodestruction. By means of quenching experiments it was deduced that this was mainly due to the sensitized production of singlet oxygen, but also to some extent to the sensitized formation of superoxide radicals which then react with bilirubin IX$\alpha$. In absence of oxygen, a photochemically induced disproportionation reaction of hypericin may lead to the oxidative degradation of bilirubin IX$\alpha$. Upon selective binding of the two pigments to human serum albumin the photosensitized bilirubin IX$\alpha$ destruction was found to be compeletely retarded. Accordingly, the presence of bilirubin IX$\alpha$ within the realm of hypericin assisted photodynamic therapy should have no adverse effects, and neonatal jaundice phototherapy would not benefit from an administration of hypericin.

**Keywords.** Hypericin; Bilirubin; Human serum albumin; Photooxidation.

**Zur hypericinsensibilisierten Photooxidation von Bilirubin IXα**

**Zusammenfassung.** Für belüftete protische Lösungsmittel, wie Ethanol oder 80% wäßriges Ethanol, wurde gefunden, daß Bilirubin IX$\alpha$ einem hypericinsensibilisierten oxidativen Photoabbau unterliegt. Mit Hilfe von Quenchexperimenten wurde abgeleitet, daß dies hauptsächlich auf die Bildung von Singlettsauerstoff und in gewissem Ausmaß auch auf die Bildung von Superoxidradikalen zurückzuführen ist, welche dann mit Bilirubin IX$\alpha$ reagieren. In Abwesenheit von Sauerstoff führt offenbar eine photochemisch induzierte Disproportionierung von Hypericin zum oxidativen Abbau von Bilirubin IX$\alpha$. Werden die beiden Pigmente selektiv an Humanserumalbumin gebunden, wird die photosensibilisierte Zerstörung des Bilirubins IX$\alpha$ vollständig verhindert. Dementsprechend sollte die Gegenwart von Bilirubin IX$\alpha$, wie sie unter den Bedingungen der Phototherapie mit Hypericin gegeben ist, keinen ungünstigen Einfluß ausüben. Hypericingaben für die Phototherapie der Gelbsucht Neugeborener wären demgemäß aber nicht sinnvoll.

## Introduction

Hypericin (**1**), or more precisely, its 3-phenolate ion, is a natural photosensitizing polycyclic quinone. It displays significant virucidal activity and possesses antiproliverative and cytotoxic effects on tumor cells [1]. The mechanism of

[a] Dedicated to Prof. Dr. *K. Schlögl* on the occasion of his 75th birthday

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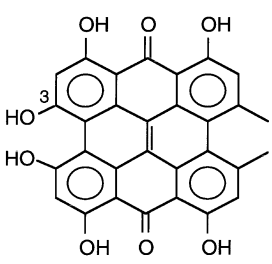
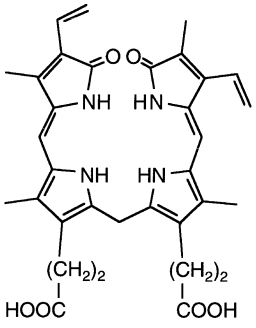
photodynamic therapy involving **1** could be mainly due to three possible mechanisms. First, formation of singlet oxygen ($^3\mathbf{1}+O_2 \rightarrow \mathbf{1}+{}^1O_2$) with a quantum yield in the order of 0.4 has been observed to originate from the excited hypericin triplet state [2]. This reaction involving radiative transfer has to be classified as type II of photosensitized reactions [3]. Second, the superoxide radical can be produced also in a reaction of the hypericin triplet with molecular oxygen ($^3\mathbf{1}+O_2 \rightarrow \mathbf{1}^{\cdot+}+O_2^{\cdot-}$) [4, 5]. This is a photo-sensitized reaction of type I [3] involving electron transfer. The two oxygen species will then exert their deleterious actions upon the target organisms or cells. Third, photoejection of protons originating from excited state processes could take place. These could then induce acidification of the organisms or cells, thereby possibly causing apoptosis [6, 7].




**1**




**2**

Under physiological conditions, serum albumin will form strong selective heteroassociates with **1** which is favorably bound ($K_f = 7.5 \times 10^4\, M^{-1}$ [8]) to the active site of the subdomain IIIA [9]. However, under physiologic conditions, bilirubin IX$\alpha$ (**2**) will also be present, and as with **1** it will be mostly found selectively bound ($K_f \approx 10^7$) to the active site of the subdomain IIA of serum albumin [10, 11]. The two binding regions are closely spaced [10, 12]; it may therefore be anticipated that if the two pigments are simultaneously bound to albumin they could interact upon the influence of light. Since **2** is known to vividly undergo reactions with singlet oxygen or radicals of **1** [13], this could have implications for the use of **1** in phototherapy and photdynamic therapy. Hence, we studied the hypericin sensitized photchemistry of bilirubin without and in presence of oxygen and human serum albumin.

## Results and Discussion

Before studying the photochemical interaction reactions of the two pigments **1** and **2**, they were investigated with respect to ground state interactions. By means of UV/Vis difference spectroscopy of ethanolic or aqueous ethanolic (80%) solutions of equimolar amounts of the sodium salt of **1** and the disodium salt of **2** before and after mixing in a tandem cuvette, virtually no spectroscopic changes could be detected, and accordingly no indication of the formation of ground state heteroassociates between the anions of **1** and **2** were obtained.

Since it is well known that bilirubin IX$\alpha$ (**2**) is photodestructed *via* dye sensitized and self sensitized mechanisms to produce biliverdin IX$\alpha$, propentdyo-

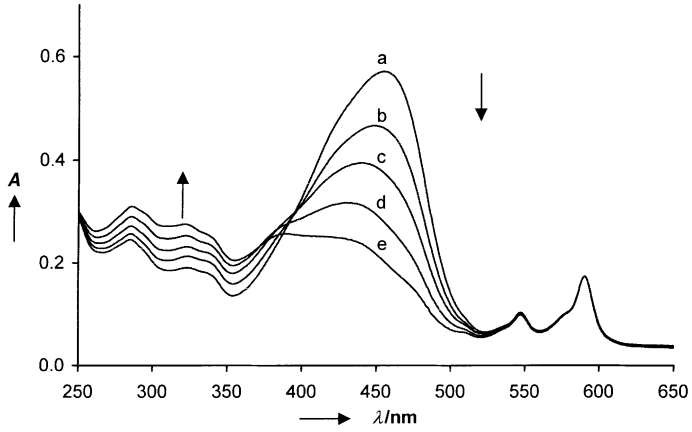



**Fig. 1.** Absorption spectra of a solution of sodium hypericinate ($^{(3-)}$**1**; $c = 3.5 \cdot 10^{-6}$ mol · dm$^{-3}$) and disodium bilirubinate (**2**$^{--}$; $c = 8.2 \cdot 10^{-6}$ mol · dm$^{-3}$) in aerated 80% aqueous ethanol before (a) and after irradiation at $\lambda > 570$ nm for 60, 120, 180, and 240 seconds (b–e)

pents, and several imides [13, 14], this reaction can be easily monitored by the disappearance of the absorption band of **2** at about 450 nm. The main absorption band system of **1** is nicely separated from that of **2** with the main absorption band centered at about 590 nm.

In Fig. 1 the absorption spectrum of a mixture of the sodium salts of **1** and a 2.3-fold molecular excess of **2** dissolved in 80% ethanol is shown. When irradiated with light of $\lambda > 570$ nm, the aerated solution displayed a loss of the bilirubin absorption band intensity as shown in Fig. 1. This loss was found to be linearly correlated with the irradiation time. In this experiment the only spectroscopic changes were observed within the bilirubin absorption band which diminished in intensity, and within the wavelength region below 400 nm, where an increase of intensity pointed to the accumulation of the oxidative degradation products of bilirubin. However, in the wavelength region above the bilirubin absorption band absence of spectroscopic changes pointed to the fact that neither **1** underwent any reactions nor biliverdin IX$\alpha$, which could in principle be formed by oxidation of **2**, accumulated.

The preceding experiment is also illustrated in the form of trace b in the normalized graph of Fig. 2 together with the resulting trace a of two experiments in which either no irradiation was applied or the solution containing only **2** was irradiated with light of $\lambda > 570$ nm. This latter experiment verified that the spectroscopic changes observed in the experiment given in Fig. 1 and as trace b in Fig. 2 were indeed due to a hypericin sensitized photodegration of **2**.

To explore the role of oxygen in this process, a carefully degassed solution of the sodium salts of **1** and a 2.2-fold molecular excess of **2** was prepared and irradiated as described above. Trace c in Fig. 2 clearly shows that the bilirubin degradation is not as efficient as in the presence of oxygen. Nevertheless, destruction of **2** took also place to a significant degree. Using absolute ethanol in the experiments described above led to a similar decrease (*e.g.* Fig. 2, trace b) in the destruction rates as compared to the 80% aqueous ethanol solutions; however, essentially the same qualitative results were obtained.

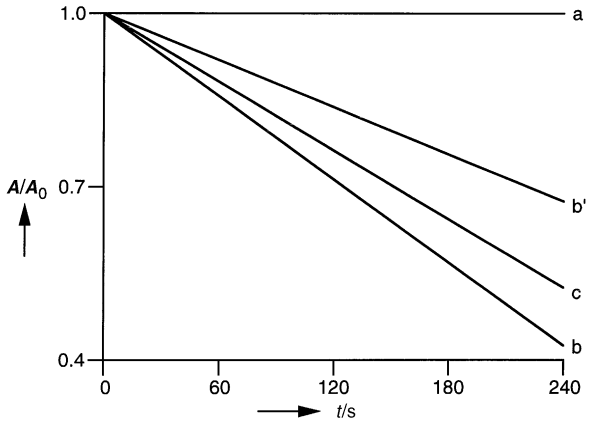



**Fig. 2.** Normalized absorption changes ($A/A_0$) with time of a solution of sodium hypericinate ($^{(3-)}\mathbf{1}$; $c = 3.5 \cdot 10^{-6}\,\mathrm{mol \cdot dm^{-3}}$) and disodium bilirubinate ($\mathbf{2}^{--}$; $c = 8.2 \cdot 10^{-6}\,\mathrm{mol \cdot dm^{-3}}$) at $\lambda = 450\,\mathrm{nm}$ in aerated 80% aqueous ethanol upon no irradiation or irradiation at $\lambda > 570\,\mathrm{nm}$ of the given solution containing only **2** (a), irradiation at $\lambda > 570\,\mathrm{nm}$ (b), using aerated absolute ethanol as the solvent for the pigments and irradiation at $\lambda > 570\,\mathrm{nm}$ (b'), and irradiation and the corresponding degassed solution in 80% aqueous ethanol with irradiation at $\lambda > 570\,\mathrm{nm}$ (c)

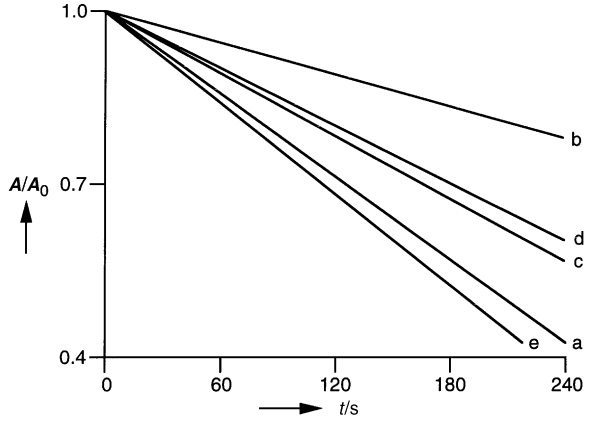



**Fig. 3.** Normalized absorption changes ($A/A_0$) with time of a solution of sodium hypericinate ($^{(3-)}\mathbf{1}$; $c = 1.4 \cdot 10^{-6}\,\mathrm{mol \cdot dm^{-3}}$) and disodium bilirubinate ($\mathbf{2}^{--}$; $c = 4 \cdot 10^{-6}\,\mathrm{mol \cdot dm^{-3}}$) at $\lambda = 450\,\mathrm{nm}$ in aerated 80% aqueous ethanol upon irradiation at $\lambda > 570\,\mathrm{nm}$ (a) and upon irradiation at $\lambda > 570\,\mathrm{nm}$ with sodium azide (b), 1,4-diazabicyclo[2.2.2]octane (c), benzoquinone (d), and iodomethane, in this case in absolute ethanol (e), added

To address the question of mechanism of the observed hypericin senesitized bilirubin photodestruction, typical quenchers were added before the photo reaction was executed. From Fig. 3 (trace b) and a comparison with the parent system (trace a) it followed that the singlet oxygen quencher sodium azide [15] very effectively quenched the reaction suggesting that hypericin induced singlet oxygen production; accordingly, a type II photosensitized reaction is mainly involved in the reaction process. Another singlet oxygen quencher, 1,4-diazabicyclo[2.2.2]octane [15] (Fig. 3, trace c), which is known to quench singlet oxygen *via* a charge

separation process in protic solvents, displayed a much less pronounced quenching effect. However, this did not rule out the type II mechanism, because the high polarity of the solvent (the quenching is more pronounced in absolute ethanol than in 80% ethanol) might cause the charged species to separate producing the superoxide radical which then could cause bilirubin degradation. Similar quenching effects were observed using the superoxide radical quencher benzoquinone [16] (Fig. 3, trace d). This result together with the preceding one indicated that obviously part of the reaction could also take place *via* a type I sensitized photodestruction involving the superoxide radical. Addition of iodomethane increased the photooxidation rate as demonstrated by trace e of Fig. 3. It should be noted that due to incomplete miscibility 96% ethanol had to be used in this experiment. Accordingly, this trace has to be compared with trace b of Fig. 2, which, of course, reveals that the effect indeed is a rather large one. It might be due to the heavy atom effect which either may prolong the singlet oxygen life time or may enhance the intersystem crossing quantum yield of **1**.

Taken together the experiments described above indicate that the hypericin photosensitized oxidative photodegradation of bilirubin IX$\alpha$ (**2**) in protic solvents may involve at least three different mechanisms. In presence of molecular oxygen, the hypericin sensitized formation of singlet oxygen according to $^3\mathbf{1}+O_2 \rightarrow \mathbf{1}+{}^1O_2$ [2] will take place as the predominating process. In addition, formation of the superoxide radical according to $^3\mathbf{1}+O_2 \rightarrow \mathbf{1}^{\cdot+}+O_2^{\cdot-}$ [4, 5] will occur, thus following the type I and II mechanisms of photosensitized reactions. These two species will then oxidatively degrade **2** in a well established [13, 14] way. In absence of molecular oxygen the photochemically induced disproportionation $^3\mathbf{1}+\mathbf{1} \rightarrow \mathbf{1}^{\cdot-}+\mathbf{1}^{\cdot+}$ [17] was regarded to constitute the starting reaction of a radical mediated oxidative degradation of bilirubin (**2**).

When the sodium salt of **1** was bound to an equimolar amount of human serum albumin together with a slightly smaller than equimolar amount of the sodium salt of **2**, and this system was dissolved in an aerated aqueous phosphate buffer at

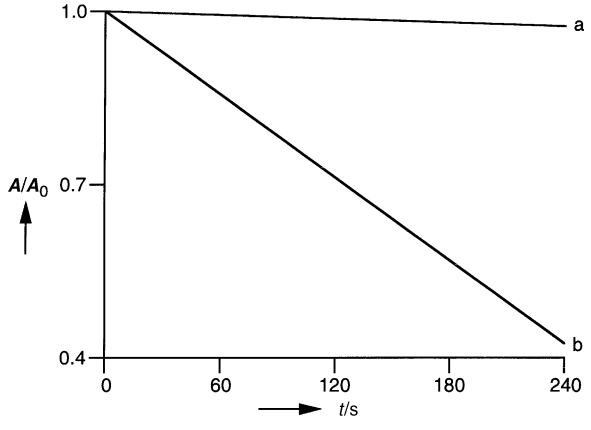



**Fig. 4.** Normalized absorption changes ($A/A_0$) with time of the complex of sodium hypericinate ($^{(3-)}\mathbf{1}$; $c = 2.8 \cdot 10^{-5}\,\mathrm{mol \cdot dm^{-3}}$) and disodium bilirubinate ($\mathbf{2}^{-}$; $c = 2.5 \cdot 10^{-5}\,\mathrm{mol \cdot dm^{-3}}$) at $\lambda = 450\,\mathrm{nm}$ with human serum albumin ($c = 2.8 \cdot 10^{-5}\,\mathrm{mol \cdot\ dm^{-3}}$) in aerated phosphate buffer ($pH = 6.9$) upon irradiation at $\lambda > 570\,\mathrm{nm}$ (a) and trace b of Fig. 2(b)

$pH = 6.9$, virtually no hypericin sensitized photodegradation of bilirubin IX$\alpha$ (**2**) could be detected (Fig. 4, trace a). Although the two pigments are attached to the active sites of two rather closely spaced subdomains of the albumin [9–11] and also a much higher amount of the sensitizer pigment as compared to the experiments in homogeneous solutions described above was present, neither type I nor type II photo reactions became operative. Thus, on the one hand, bilirubinate is effectively protected by the surrounding protein from hypericin sensitized photodegradation. On the other hand, the hypericinate ion might also be shielded from producing deleterious agents that could harm the bilirubinate.

### *Conclusions*

In contrast to aerated homogeneous solutions in protic solvents where mainly type I but also type II hypericin (**1**) photosensitized oxidative photdestruction of bilirubin IX$\alpha$ (**2**) is encountered, a photochemical interaction between **2** and **1** under physiological conditions, *i.e.* in presence of serum albumin, can be neglected. Accordingly, within the realm of photodynamic therapy no measures to account for such an interaction will be necessary to be taken. With respect to the phototherapy of neonatal jaundice [18], application of **1** with the aim to promote oxidative bilirubin degradation in addition to the photodiastereomerization process would seem to present no advantage. However, viewed from the perspective of the benefiting role **2** may exert as a defence of the body against oxidants and radicals [19], administration of **1** in the course of photodynamic therapy should not cause serious problems.

## Experimental

Hypericin (**1**) was prepared and purified according to Refs. [20, 21]. Bilirubin was of commercial origin (Sigma) and was purified according to the procedure of *McDonagh* [22]. The corresponding sodium salts of **1** and **2** were prepared by titration of their methanolic solutions with NaOH and evaporation of the solvent. As solvents absolute ethanol (p.a. Merck) and 80% aqueous ethanol (p.a.) were used.

Aerated solutions were prepared by bubbling with air for 10 min prior to use. Degassing of solutions was achieved by first bubbling with Ar for 10 min and then repeating a freeze-pump-thaw cycle four times. Sodium azide (p.a. Merck), 1,4-diazabicyclo[2.2.2]octane (Fluka), and benzoquinone (Merck) serving as the quenchers and iodomethane (Merck) were of commercial origin. They were used as obtained and added to the pigment solutions to yield concentrations of about $10^{-3}\,\mathrm{mol \cdot dm^{-3}}$. The albumin complex was prepared *in situ* by first adding an equimolar amount of the sodium salt of **1** dissolved in a minimum amount of 0.1 *M* KOH to a solution of serum albumin (97–99%, 1× crystallized and lyophylized; Sigma) in phosphate buffer ($pH = 6.9$). After standing for several hours (the complexation was monitored by means of CD spectra recorded on an ISA Autodichrograph, Mark V, cf. [9]), an equimolar amount of the disodium salt of **2** dissolved in a minimum amount of 0.1 *M* KOH was added. The completion of the complexation was again monitored by means of CD Spectra, in which the two bound pigments displayed chiroptical signals as described in literature [9, 23]. All experiments were strictly executed under subdued light.

Irradiations of the thermostatted samples ($25 \pm 1^{\circ}\mathrm{C}$) contained in $SiO_2$ cuvettes ($d = 1$ cm) were performed using a 300 W tungsten lamp and a cut-off filter effectively blocking light below 570 nm. UV/Vis spectra were recorded with a Hewlett Packard 8453 photodiode array spectrometer.

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