PHOTO-OXIDATION OF BILIRUBIN

by J.C. de Paula

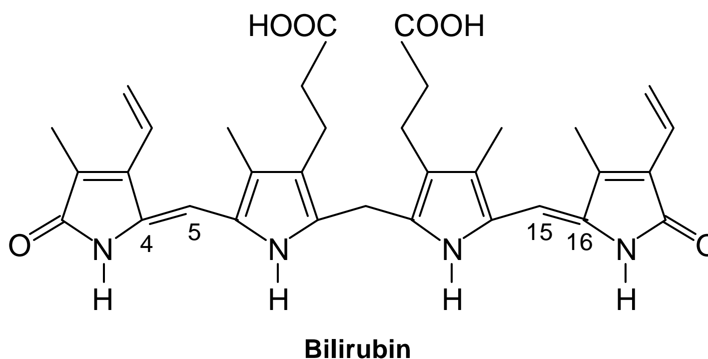
1. PURPOSE OF THE EXPERIMENT

You will investigate a photochemical reaction involving the photosensitization of singlet O_2 in solution. The photosensitizer will be a synthetic porphyrin and the reaction will lead to the oxidation of bilirubin. You will also investigate the inhibition of singlet O_2-mediated photochemistry by β-carotene.

I acknowledge extensive discussions with Prof. Valerie Walters on ways to implement and improve this experiment, which is based on a published procedure.[1]

2. INTRODUCTION

Bilirubin, shown below, is present in millimolar quantities in blood and is produced continuously as a result of breakdown of hemoglobin by the enzyme heme oxygenase. It has been proposed that bilirubin is a natural anti-oxidant that can help the body fight a variety of biochemical stresses. However, when bilirubin levels are too high, a potentially dangerous condition known as jaundice may result. If left untreated, jaundice may cause irreversible neurological damage in newborn babies.



Bilirubin

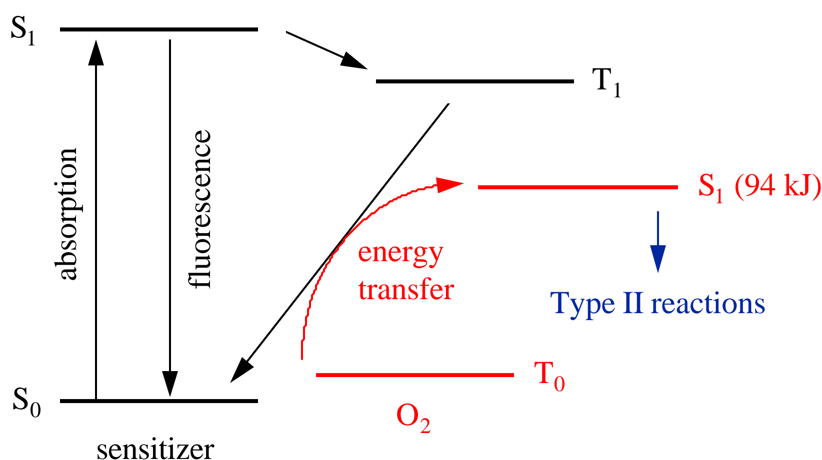
Bilirubin undergoes several photochemical reactions when irradiated with light of about 450 nm. It can undergo a configurational (cis-trans) isomerization about the C_4-C_5 or C_{15}-C_{16} double bonds. When bound to serum albumin (as it is naturally in the blood), absorption of light causes a

[1] Photobiological Techniques, D.P. Valenzeno et al., Eds., Plenum Press, New York, 1991, Chapter 5.

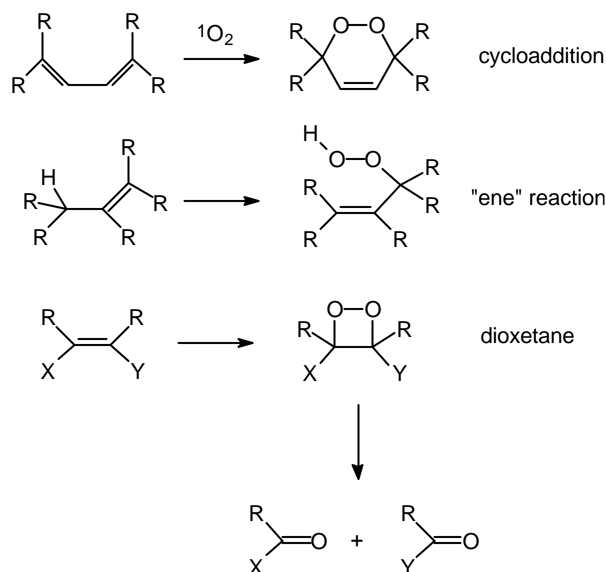
structural isomerization to form lumirubin; the reaction involves an intramolecular cyclization. A third photochemical reaction is photooxidation.

Bilirubin can be photooxidized by a highly reactive form of O_2, known as singlet O_2. Singlet O_2 is molecular oxygen in a high-energy excited state in which all electron spins are paired (that is, a singlet state). Recall that the ground state of O_2 is a triplet state, meaning that there are two unpaired electron spins.

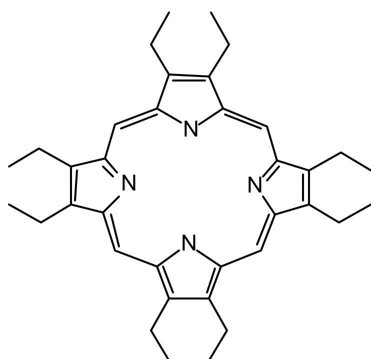
Singlet O_2 is typically formed by the mechanism depicted below. Energy in the form of light is absorbed by a photosensitizer to form an excited singlet state which then decays to an excited triplet state (T_1 state). The excited photosensitizer then transfer its energy to ground state O_2, resulting in the formation of singlet O_2. In turn, the photochemically generated singlet O_2 oxidizes a variety of substrates in what is known as a *photodynamic type II reaction*.



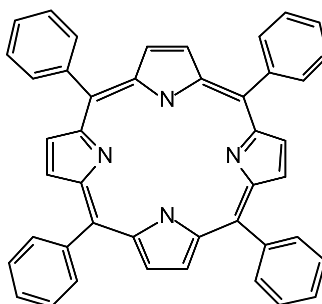
Some of the oxidation reactions in which singlet O_2 ($^1O_2$) can participate are shown below.



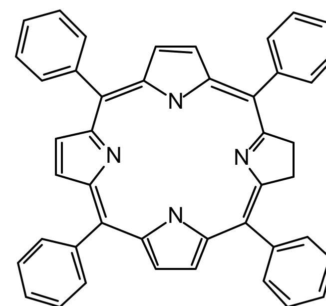
Porphyrins, such as octaethylporphine (H_2OEP, shown below), are excellent photosensitizers of singlet O_2. In this experiment, you will compare the photosensitizing efficiencies of H_2OEP, H_2TPP, H_2TPC, and $H_2TFPPBr_8$. The structures of the first three are shown below. $H_2TFPPBr_8$ has a structure similar to H_2TPP except that each of the four phenyl rings have five fluorine substituents and each of the four pyrrole rings have two bromine substituents located on the outside of the porphyrin ring.



octaethylporphine
(H_2OEP)

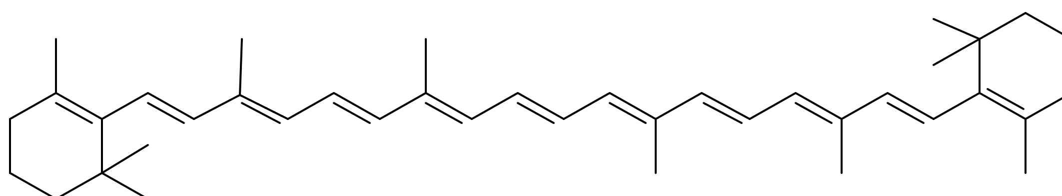


meso-tetraphenylporphine
(H_2TPP)



meso-tetraphenylchlorin
(H_2TPC)

A number of experiments are required to prove that photochemical degradation of bilirubin is both photosensitized by a porphyrin and involves singlet O_2. First, it is necessary to irradiate a solution containing only bilirubin. Some photodegradation is expected in this case (see above). Second, a solution containing both porphyrin and bilirubin is irradiated. Enhanced degradation of bilirubin under these conditions proves that the porphyrin is somehow involved but does not necessarily implicate singlet O_2 as the oxidant. It is possible, for example, that a light-induced electron transfer reaction is responsible for the oxidation of bilirubin, leaving the porphyrin reduced. Finally, the role of singlet O_2 in the photodegradation mechanism can be assessed by irradiating a solution that contains porphyrin, bilirubin, and a compound that scavenges singlet O_2 in solution. For example, singlet O_2 transfers energy very efficiently to β-carotene (shown below) before it has a chance to oxidize other substances. Therefore, inhibition of photochemistry in the presence of β-carotene is strong evidence for the involvement of singlet O_2 in the mechanism.



β-Carotene

3. PROCEDURES

You will be instructed on the use of the Ocean Optics spectrophotometer and the photolysis source. This spectrophotometer is capable of automatically monitoring peak absorption at set time intervals.

Except where noted, these experiments should be done in the dark or under subdued lighting. Solutions containing bilirubin should be protected from light or manipulated under an orange or red safelight.

List of materials.

- Bilirubin (Porphyrin Products);
- Porphyrins (Aldrich or Porphyrin Products);
- β-Carotene (Sigma);
- Chloroform (spectrometric grade)
- Acetone wash bottle;
- Cuvettes (two per group);
- 10 mL volumetric flasks;
- 1 mL volumetric pipettes and pipette bulb;
- Pasteur pipettes;
- Stopwatch;
- Photolysis setup (ORIEL 77503 fiber-optic illuminator coupled to an ORIEL 77612 collimator)
- Ocean Optics single-beam spectrometer with CCD detector.

Preparation of solutions.

- 8.8 mg of bilirubin dissolved in 100 mL of chloroform (solution A).
- 1.0 mg of H_2OEP dissolved in 100 mL of chloroform (solution B1). What is the concentration of porphyrin in this solution?
- 1.0 mg of H_2TPP in 100 mL of chloroform (solution B2). What is the concentration of porphyrin in this solution?
- 1.0 mg of H_2TPC in 100 mL of chloroform (solution B3). What is the concentration of porphyrin in this solution?
- 1.0 mg of $H_2TFPPBr_8$ in 100 mL of chloroform (solution B4). What is the concentration of porphyrin in this solution?
- 8.1 mg of β-carotene dissolved in 100 mL of chloroform (solution C).

Control photooxidation. Perform this experiment once.

- Dilute solution A ten-fold with chloroform in a volumetric flask and transfer 3.5 mL to a cuvette.
- Record the absorbance of this solution versus a chloroform blank at 450 nm.
- Place the cuvette containing the sample solution immediately in front of the photolysis setup. Set up the spectrometer for automatic monitoring of 450 nm absorption at 10 sec time intervals up to a total of 10 minutes.
- Discard the contents of the cuvettes (into the organic waste container in the hood). Rinse the cuvettes with acetone and blow them dry in a slow stream of dry air.

Porphyrin-sensitized photooxidation. Perform this experiment once for each porphyrin (solutions B1, B2, B3 and B4).

- Dilute solution B1, B2, B3, or B4 ten-fold with chloroform. This is your blank solution.
- Mix 1.0 mL of solution A and 1.0 mL of solution B1, B2, B3, or B4 in a 10 mL volumetric flask and make the volume of the solution up to 10 mL with chloroform.
- Transfer 3.5 mL of this solution to the sample cuvette and record the absorbance at 450 nm as before.
- Expose the sample solution to light from the photolysis setup and record the absorbance at 450 nm at the same intervals as above.
- Discard the solution into the organic waste container. Rinse and dry the cuvettes as before.

Inhibition by β-carotene. Perform this experiment once for each porphyrin.

- Mix 1.0 mL of solution B1, B2, B3, or B4 and 1.0 mL of solution C in a volumetric flask and make the volume of the solution up to 10 mL with chloroform. This is your blank solution.
- Mix 1.0 mL of solution A, 1.0 mL of solution B1 or B2, and 1.0 mL of solution C in a volumetric flask and make up to 10.0 mL with chloroform.
- Transfer 3.5 mL of this solution to the sample cuvette and record the absorbance at 450 nm.
- Expose the solution to light from the photolysis setup and record the absorbance at 450 nm at the same intervals as before.
- Discard the solutions and rinse and dry the cuvettes as before.

Absorption Maxima of Porphyrins. Obtain the uv-vis absorption spectrum of each of the porphyrin solutions and record the wavelength maximum of the "reddest" peak.

4. DATA ANALYSIS

Plot % decrease in absorbance at 450 nm versus illumination time for all experiments. You do not need to fit the data to an equation. Prior to writing your laboratory report, please turn in a data summary consisting of: (i) plots showing time courses for all experiments; and (ii) a table summarizing the sensitizing efficiency of each porphyrin (as % decrease in absorbance after 10 min of illumination).

5. THE LABORATORY REPORT

The report on porphyrin photochemistry will be full-length and written according to the guidelines in the *General Remarks* document available from the course's web site. This report will incorporate the results of your literature search and of your photochemistry experiments. Here are the principal elements of the report:

Title. A title followed by the names of the students in your group. Your name should be followed by an asterisk.

Abstract. Please include the following: (a) a one or two sentence description of the main goal of the study; (b) a one or two sentence summary of the data; and (c) a one or two sentence summary of your conclusions, along with the identification of the most efficient sensitizer in your group of compounds.

Introduction. Give background on the nature of your study. This is your chance to include information that you obtained via the on-line literature search.

Experimental Methods. Do not rewrite laboratory handouts. Refer to them as much as possible to give the reader a sense of your experimental strategy and do include modifications of the procedure.

Results. Show a single plot of % decrease in absorbance versus illumination time where data for all porphyrins and the control are displayed simultaneously. This allows for quick visual comparison of the relative photosensitizing efficiencies. Also provide a table summarizing the sensitizing efficiency of each porphyrin, normalized for the value of % decrease in absorbance after 10 min of illumination that you measured for the most efficient porphyrin.. Please write a bit of prose to accompany the figures and tables. Finally, discuss the effect of β-carotene on the photochemical experiments.

Discussion. Please propose a mechanism for the photo-oxidation of bilirubin and for the inhibition by β-carotene. Do your data suggest that singlet O_2 is involved in the photooxidation of bilirubin? Comment on which porphyrin is a better photosensitizer. "Better" can have several meanings, so please consider as many of these meanings as possible. Justify your statement with your observations and references from the literature. You will not be able to explain exactly why one porphyrin is more efficient than the other. However, using knowledge from your literature search, you will be able to propose experiments that may explain the trends you observed. Please do so. Finally, comment on possible sources of error in this experiment and ways in which they can be minimized.

Acknowledgments.

References. Please follow the format described in the *General Remarks* section of the lab manual.