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KINETICS OF THE FORMATION OF BILIVERDIN DURING THE PHOTOCHEMICAL OXIDATION OF BILIRUBIN MONITORED BY COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

In the photochemical oxidation of bilirubin, biliverdin is formed as the primary product and is further degraded This photooxidation is especially significant in the presence of riboflavin Column liquid chromatography was used to monitor the kinetics of this reaction The biliverdin concentration amounts to a maximum of ca 38% of the total loss of bilirubin in experiments in vitro It is probable that this mechanism is also operative during phototherapy The formation of a product of the photooxidation of biliverdin that has not yet been identified has been observed, the product behaves as a dimer A method for the determination of biliverdin in the blood of newborn infants has been developed It has been found that the biliverdin content increases during hyperbilirubinaemia

INTRODUCTION

Biliverdin is an important intermediate in the haeme degradation by microsomal elements, attributed to a system of oxygenases consisting of nicotinamide-adenine dinucleotide phosphate (reduced, NADPH) cytochrome c reductase, haeme oxygenase and biliverdin reductase, the products being biliverdin IX_α, bilirubin IX_α and various fractions of the tetrapyroole skeleton of bilirubin [1—5]. The bilirubin metabolism is important for newborn infants, as it can be connected with damage of their brain tissue.

In the photochemical degradation of bilirubin in vitro, photooxidation occurs, accompanied by chemical changes in the bilirubin molecule, as well as isomerization Bonnett and Davis [6] first described bilirubin isomerization, while Cohen and Ostrow [7] pointed out its importance for phototherapy McDonagh et al. [8] studies the formation of photoisomers and their structure,

both in vitro and in vivo. A change in the configuration occurs on the exocyclic double bonds of bilirubin, from the basic structure (4-Z, 15-Z) to a mixture of E and Z isomers with the predominating 4-Z, 15-E structure. The principal work on the photooxidation of bilirubin is that of Bonnett and Stewart [9], the authors isolated biliverdin and some dipyrrole derivatives as the products The topic was critically reviewed by Ostrow [10] and recently by Lightner et al [11] who determined the products of photooxidation of bilirubin in the urine of newborn infants by liquid chromatography (LC) The formation of biliverdin in the photooxidation of bilirubin was pointed out by Bonnett and Stewart [9], Gray et al [12], Ostrow and Nicholson [13], and Knobloch and co-workers [14, 15]

The photooxidation of bilirubin is catalysed by various activators, whose use was discussed by Cremer and Perryman [16]. The photochemical degradation of bilirubin was studied in vitro, in the presence of riboflavin, by Sanvordecker and Kostenbauder [17] who explain the mechanism of the activator effect by the formation of singlet oxygen The mechanism of the reaction was further studied by Knobloch and co-workers [14, 15] under aerobic and anaerobic conditions. The authors assume that the reaction mechanism involves a reversible reaction between bilirubin as an electron donor and riboflavin in the triplet state as an electron acceptor, according to the scheme assumed for the function of flavine in redox systems [18] This scheme does not assume participation of singlet oxygen In the following years, this participation was also excluded in the works of Landen et al [19] and Kusuki and Enoke [20]. Meisel and Jähng [21] state that the presence of riboflavin causes a change in the intermediate ratio in the photochemical degradation of bilirubin in the presence of albumin, so that photooxidation is enhanced at the expense of isomerization New possibilities for the study of the degradation of bilirubin by light have been provided by the use of LC. The method was first used by Lim et al. [22] for the determination of unconjugated bilirubin in serum Cole and Little [23] described an LC determination of bilirubin in the dog gall and a determination of biliverdin isomers. Uesugi et al. [24] determined conjugated derivatives of bilirubin, whereas the determination of the photoisomer was studied by McDonagh et al. [8] and Onishi and coworkers [25, 26] Yoshinaga and Sassa [5] described a determination of biliverdin isomers. O'Carra and Colleran [27] employed thin-layer chromatography (TLC) for identification of biliverdin isomers. They also used LC as a very sensitive and specific method for the study of the mechanism of the formation of biliverdin in vitro during the photochemical oxidation of bilirubin and for the determination in the blood of newborn infants.

EXPERIMENTAL

The bilirubin substance (Merck, Darmstadt, F R.G.) and a lyophilized preparation with albumin (Laboratorní potřeby, Prague, Czechoslovakia, 0.8 mg of bilirubin per 80 mg of bovine albumin) were used As the riboflavin preparation, a riboflavin-5-phosphate salt (Hoffmann la Roche, Basel, Switzerland) that is readily soluble in water, was employed. Biliverdin was prepared by the modified method of Lemberg [28] a bilirubin suspension in concentrated acetic acid was oxidized at $80°C$ (5 mg of iron(III)chloride per 3 mg of

bilirubin), the mixture was diluted with water after 3 h, extracted with diethyl ether and the extracts were washed with water containing 2 M sodium acetate. A standard preparation was obtained by chromatography on Florisil with elution by methanol The purity was checked by TLC on a silica gel layer (Kieselgel 60, Merck) in chloroform—acetone—methanol—acetic acid (80 10.9 1) using the method modified for monitoring of the free forms, according to the original work of O'Carra and Colleran [27] who, however, used dimethylesters of biliverdin The predominating isomer is biliverdin IX_α. As the source of radiation, a halogen discharge lamp used in phototherapy was employed, radiating at 425—475 nm with an output of 6.2 $\mu W/cm^2/nm$ [29], at a distance of 80 cm.

The absorption spectra were obtained on a Unicam SP 800 instrument and the bilirubin concentration was calculated from the absorbance at 460 nm (ϵ = 56 000) in an aqueous medium containing albumin and that of biliverdin from the absorbance at 650 nm (ϵ = 15 100) in methanol.

The LC analysis was performed on a PU 4020 Pye Unicam instrument with optical detection from 220 to 400 nm, a Spherisorb ODS-5 RP-18 (surface octadecyl-silan, particle size 5 μm) (100 X 4.6 mm I D) column, Pye Unicam. Mobile phase Tris buffer containing 80% methanol and 0 01 M phosphoric acid at pH 4 2, adjusted with hydroxymethylaminomethane. A similar system with phosphoric acid was also used by Cole and Little [23]

The solutions were irradiated in Petri dishes in a medium of 0 01 M phosphate buffer (pH 6 7—7.3), the layer thickness amounting to 1 cm. The photodegradation of bilirubin was followed spectrophotometrically and, simultaneously, samples were collected for the determination of biliverdin To 2-ml samples, 1 ml of 0.2 M succinic acid and 1 ml of 20% sodium chloride solution were added, the biliverdin was extracted into diethyl ether, the extract was evaporated and the residue dissolved in 1 ml of the elution solution and injected directly into the colum. Determination in the blood serum was carried out after denaturization of proteins by acidified methanol according to Gray et al [12] (5 ml of conc. hydrochloric acid per 100 ml of methanol). To 0 5 ml of plasma, 2 ml of acidified methanol were added, followed by 0.5 ml of 1 M hydrochloric acid after 15 min, 1 ml of 20% sodium chloride solution and 4 ml of chloroform. The mixture was shaken, centrifuged at 3000 g, filtered through a resistant filter and injected into the column A calibration curve was used prepared using the standard preparation. As a control substance, iron porphyrin was used, as it occurs in virtually all blood samples as a result of haemolysis A reference sample is prepared by denaturation of 0.1 ml of blood by the procedure described for the determination in plasma

RESULTS

With the mobile phase described, the main peak is obtained with an elution time of 7.8 min According to TLC, this peak corresponds to the biliverdin IX_α isomer, three small peaks (corresponding to a total concentration of 12%) can be assigned to biliverdin isomers. A chromatogram obtained after 80 min of irradiation of a bilirubin solution with albumin in a medium of phosphate buffer (pH 6.7) is given in Fig 1. In agreement with Lim and Peters [30] we
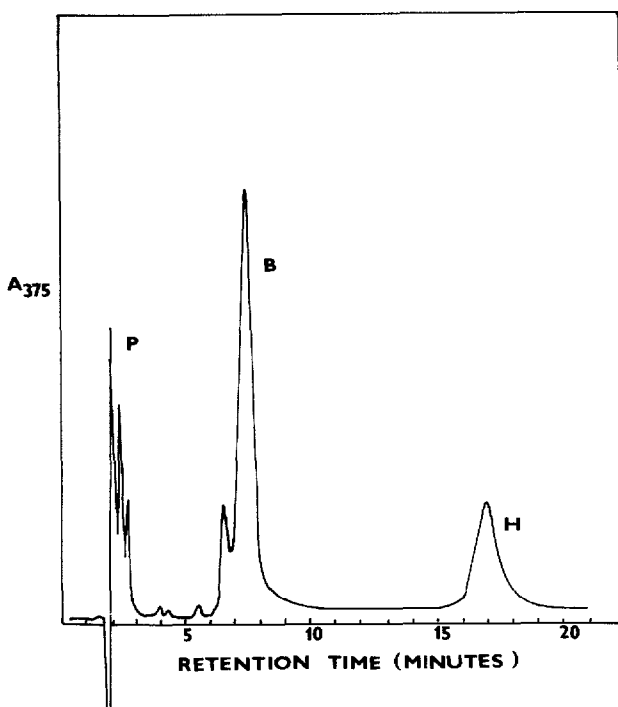
Fig 1 Chromatogram of the oxidation of bilirubin in a phosphate buffer (pH 6 7), with concentrations of 70 μmol/l bilirubin and 12 μmol/l riboflavin phosphate after 45 min of irradiation Flow-rate, 0 5 ml/min, detection at 375 nm Peaks **B** = biliverdin isomer IX_{α}, **H** = iron protoporphyrin as the reference standard

observed, when using the above elution system with Tris buffer, that the presence of the buffer substantially improves the reslution in the system Lim and Peters [30] used a methanolic solution of ammonium acetate. The zone of biliverdin is clearly visible in the figure and the time dependence of the biliverdin concentration can be readily followed during the experiment.

Fig. 2 depicts the dependence of the decrease in bilirubin concentration on time (absorbance at 460 nm), as well as the formation of biliverdin on the basicity of the LC analysis.

It can be seen from the plot that the bilirubin concentration decreased below 20% of the original value after 80 min of irradiation, while the biliverdin concentration obtained from LC increases to a maximal value that is attained in 90 min and then slowly decreases. In this stage, the solution has a green colour that further fades and turns brown—pinkish The maximum biliverdin concentration corresponds to 20 μmol, which is 30% of the initial concentration and 38% of the total loss of bilirubin From the physicochemical point of view, at this point the rates of biliverdin formation and degradation are equal The experiment clearly demonstrates that biliverdin is an important intermediate in the bilirubin degradation, even from a quantitative point of view If the experiment is carried out under identical conditions, but in the absence of riboflavin, it can be seen that destruction of bilirubin also occurs, but much more slowly, the sensitive LC method indicates that biliverdin is formed also in this
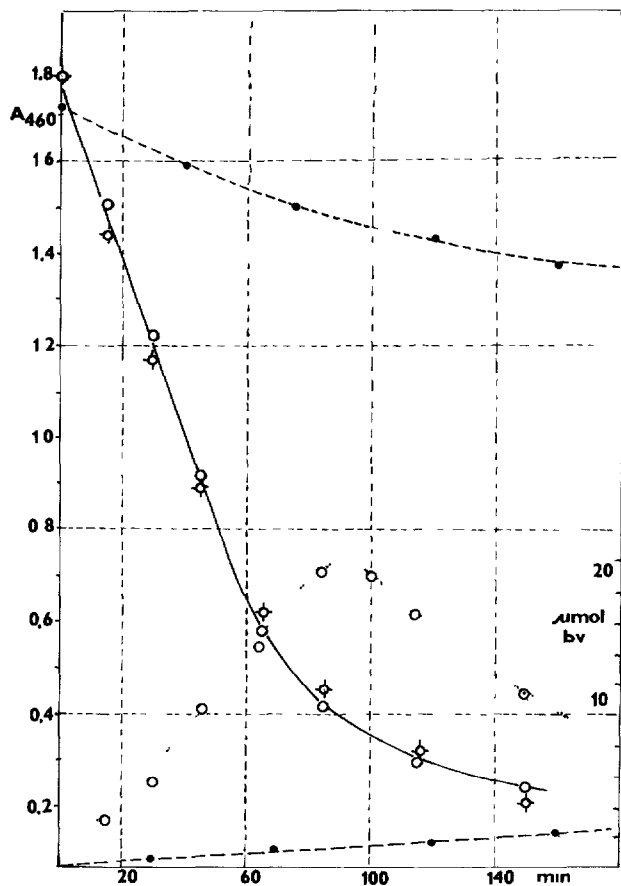
Fig 2 Photodegradation of bilirubin in the presence of riboflavin and the production of bilverdin (bv) at pH 6 7 in phosphate buffer medium The concentration of bilirubin was followed spectrophotometrically with the absorbance at 460 nm The concentration of bilverdin was measured by the LC method The initial concentration of bilirubin was 67 μmol/l (as complex with albumin) and that of riboflavin was 12 μmol/l (○) Absorbance values of experimental degradation of bilirubin, (-ȯ-) absorbance values of calculated degradation of bilirubin, (●) experimental curve of bilverdin production

case Hence, the absence of a light activator causes a decrease in the reaction rate, but photooxidation and formation of bilverdin still occur It is known that bilverdin is further degraded under aerobic conditions, an orientative experiment has shown that this reaction is also accelerated by riboflavin.

At the stage when bilirubin disappeared and the bilverdin concentration decreased to a minimum, the irradiated solution was extracted with diethyl ether and the extract was washed with a sodium acetate solution and water The irradiated solution is pink at this stage. The substances were transferred into methanol and the absorption spectrum was obtained (Fig 3), exhibiting marked absorption around 320, 516 and 545 nm. The spectrum was recorded again 30 min after an addition of 0 1 ml of 0 1 M phosphoric acid It is evident that great changes occurred and the difference spectrum indicates an increase in the absorbance at 380 and 650 nm, which corresponds to the regeneration of
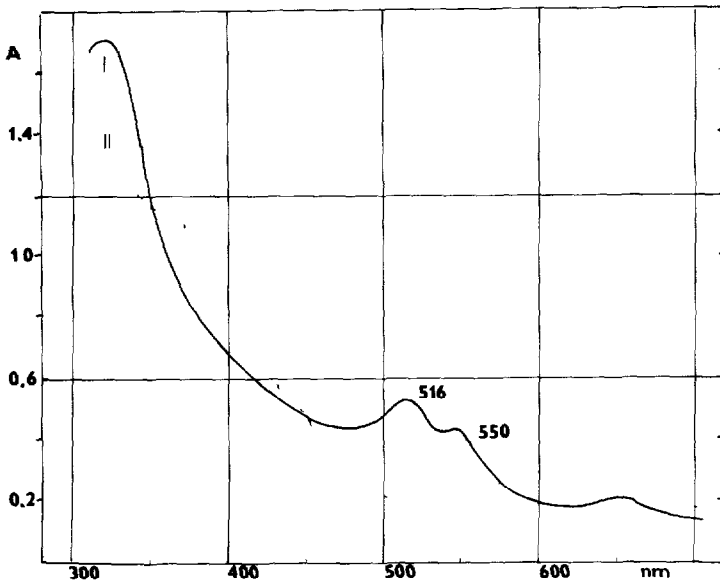
Fig 3 Absorption spectrum of the extract of the residue after irradiation of bilirubin in a phosphate buffer (pH 6 7) in methanol (———) and 30 min after addition of phosphoric acid (- - - -) using a 1-cm cuvette
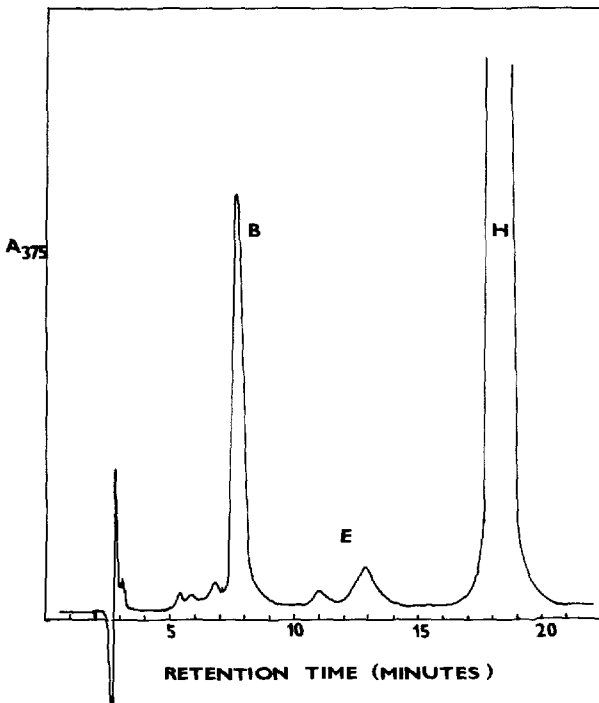

Fig 4 The determination of biliverdin in blood plasma from a female newborn, the concentration of bilirubin was 250 μmol/l and that of biliverdin was 36 μmol/l Detection at 375 nm Peaks B = biliverdin isomer IX_α, H = iron protoporphyrin

the biliverdin spectrum The monitoring of this intermediate by LC in a phosphoric acid medium is difficult because of its instability, but we succeeded qualitatively using a buffer with an organic acid (Tris buffer with succinic acid, pH 4.6)

In the determination of biliverdin in the blood of physiological newborns, we obtained relatively low values (5—8 μmol), whereas substantially higher values were obtained for newborns with an increased bilirubin content, i e for those with a metabolic defect, as can be seen from the example in Fig. 4. The female newborn had a bilirubin concentration of 250 μmol and a biliverdin concentration of 36 μmol

DISCUSSION

Photooxidation of bilirubin in the presence of riboflavin was studied earlier [14, 15, 17] Riboflavin activated by light functions as a reversible electron carrier whose reduced form is reoxidized under aerobic conditions, so that its concentration in the system can be considered constant Therefore, the conditions are satisfied for a pseudo-monomolecular reaction, described by the equation

$$kb = \frac{2\ 303}{t} \log\left(\frac{a}{a - x}\right) \tag{1}$$

where kb is the rate constant including the catalyst concentration, t is the time in min and a is the initial bilirubin concentration ($a - x$ is the loss).

The fraction found (38% of the total loss of bilirubin) is a relative value, given by the reaction kinetics, in which biliverdin is simultaneously formed and degraded, so that the actual concentration is higher. Ostrow and Branham [31] consider the qualitative identity of the products of the photochemical degradation of bilirubin and biliverdin obtained in TLC as a confirmation that biliverdin is the main intermediate in this reaction According to physicochemical criteria, the formation of an intermediate can only be monitored when the rate of the intermediate degradation is lower than that of its formation. The overall picture depends on the ratio of the two rate constants. The validity of the equation and thus the verification of the assumed mechanism were demonstrated by Experiment No 2 Constant k_1b was calculated by substituting the experimental values into eqn 1 to obtaining a mean value of 0.016 μmol/min, which was verified by the calculation from the reaction half-time In Fig. 2, the experimental ($\circ$) and the calculated (-$\diamond$-) values are compared and are expressed in terms of the absorbances at 460 nm. It can be seen that the agreement is good and a certain scatter occurs only at longer times, where the determination of the absorbance is less precise and where follow-up reactions take place.

As far as the relationship between the primary step of photooxidation and photoisomerization is concerned, photoisomerization amounts to ca 26% in vitro [32] and a maximum of 15% in vivo [33] Hence, photooxidation participates to at least the same extent as isomerization in bilirubin degradation. It is not yet known how the Z,Z and E,Z isomers are affected by photooxidation, which is probably a follow-up reaction in this system The role of

photooxidation under the conditions in vivo has been demonstrated (see above). Some products were found by Lightner et al. [11] in the urine of newborn infants. We have observed an increased secretion of biliverdin in the stool of newborns during phototherapy [34] The increased content of biliverdin during hyperbilirubinaemia observed in the present work indicates that this mechanism plays a role under pathological conditions

The pink intermediate formed during photochemical degradation of bilirubin was demonstrated by TLC in solutions of bilirubin and biliverdin irradiated in the presence of riboflavin, but also among the products formed by simple oxidation of bilirubin with iodine in a 60% acetone solution In all three systems, the same product is formed (R_F 0 38), we assume that the product is formed from biliverdin. It is probable that this is the same product as that described by Bonnett and Buckley [35] as an unknown pink substance and characterized by an R_F value in TLC. The spectral characteristics, and especially the reversible change in the spectrum owing to the effect of phosphoric acid, support our assumption that the substance is the dimer of biliverdin. Scheer and Kraus [36] consider the product of irradiation of a biliverdin derivative, 2,3-dihydroethyl-1,19-(21,24H)-bilindione-3, in the presence of ferricyanide, to be a dimer. The derivative has a similar absorption spectrum as the product obtained by us, with maxima at 325 and 574 nm, and is converted to the monomer by heat and mineral acids.

The role of this mechanism and the formation of biliverdin in vivo during hyperbilirubinaemia and phototherapy will be studied in a future work.

ACKNOWLEDGEMENTS

REFERENCES

1   R Tenhunen, H Marver and R Smith, J Biol Chem , 244 (1969) 6388
2   R Tenhunen, M Ross and H Marver, Biochemistry, 9 (1970) 293
3   B Masters, Ann Clin Res , 8 (Suppl 1) (1976) 17
4   F Guengerich, Biochemistry, 17 (1978) 3630
5   J Yoshinaga and S Sassa, J Biol Chem , 257 (1982) 7786
6   R Bonnett and E Davis, Nature, 262 (1976) 326
7   A M Cohen and D Ostrow, Pediatrics, 65 (1980) 740
8   A McDonagh, L Palma and F Trull, J Am Chem Soc , 104 (1982) 6865
9   R Bonnett and J Stewart, J Chem Soc , Perkin Trans I, 1 (1975) 224
10 D Ostrow, Bilirubin Metabolism, Blackwell, Oxford, 1967, p 117
11 D Lightner, W Linnane and Ch Ahlfors, Pediatr Res , 18 (1984) 696
12 C Gray, A Kulczycka and D Nicholson, J Chem Soc , Perkin Trans I, 1 (1972) 288
13 D Ostrow and D Nicholson, Gastroenterology, 60 (1971) 196
14 E Knobloch, R Hodr, J Herzmann and V Houdková, Acta Vitaminol Enzymol , 3 (1981) 182
15 E Knobloch, R Hodr, J Herzmann and V Houdková, Collect Czech Chem Commun , 47 (1982) 1514
16 R Cremer and R Perryman, Lancet, 1 (1958) 1094

17   D Sanvordecker and H Kostenbauder, J Pharm Sci , 63 (1974) 404
18   D Knowles and G Mautner, Photochem Photobiol , 15 (1972) 199
19   G Landen, Y Park and D Lightner, Tetrahedron, 38 (1983) 1893
20   H Kusuki and A Enoke, Photochem Photobiol , 37 (1983) 1
21   P Meisel and K Jährig, Photochem Photophys , 6 (1983) 153
22   C K Lim, V A Bull and J.M Rideout, J Chromatogr , 204 (1981) 219
23   K.D Cole and G H Little, J Chromatogr , 227 (1982) 503
24   T Uesugi, S Adachi and K Kamisaka, J Chromatogr , 277 (1983) 308
25   S Onishi and O Takahashi, Biochem J , 221 (1984) 719
26   S Onishi, M Fujikava and Y Ogawa, in J Bergsma (Editor), Bilirubin Metabolism in
     the Newborn, Excerpta of the 2nd International Symposium, Jerusalem, April, 1974,
     Elsevier, Amsterdam, 1976, p 41
27   P O'Carra and E Colleran, J Chromatogr , 50 (1970) 458
28   L Lemberg, Ann Chem , 499 (1961) 25
29   Z Baley, V Dvořáček and V Mathesius, Tesla Electron , 8 (1975) 12
30   C V Lim and I J Peters, J Chromatogr , 316 (1984) 397
31   D Ostrow and R Branham, in J Bergsma (Editor), Bilirubin Metabolism in the New-
     born, Excerpta of the 1st International Symposium, Chicago, IL, June, 1969, William
     and Wilkins, Baltimore, MD, 1970, p 93
32   J Ennever, M Sobel and A McDonagh, Pediatr Res , 18 (1984) 667
33   A Lamola nd W Blumberg, Proc Natl Sci Acad U S A , 78 (1981) 1883
34   E Knobloch, R Hodr, J Herzmann, E Procházková and V Houdková, Acta Vitaminol
     Enzymol , 6 (1984) 313
35   R Bonnett and D Buckley, Biochem J , 219 (1984) 1053
36   H Scheer and C Kraus, Photochem Photobiol , 25 (1977) 311