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Study of the mechanism of the photoisomerization and photooxidation of bilirubin using a model for the phototherapy of hyperbilirubinemia

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ABSTRACT

Liquid chromatography was employed for a study of photochemical degradation of bilirubin in the complex with human albumin, using a model system in the presence of riboflavin. The concentrations of bilirubin, the photoisomers and biliverdin were monitored. The reaction mechanism was verified using a quantified mathematical model and was represented by a reaction scheme. Photoisomerization is the initial process, followed by photooxidation to degradation products of the tetrapyrrole skeleton, with formation of biliverdin as an intermediate. The blood of newborns that were irradiated for treatment of hyperbilirubinemia was studied for the sake of comparison. The effect of some biochemically important substances was followed, in view of possible inhibition of the processes. The experimental results demonstrate that riboflavin acts as a catalyst, even at the concentrations typical for its occurrence in blood. The results are discussed from the point of view of the mechanism of bilirubin degradation during phototherapy.

INTRODUCTION

Irradiation of bilirubin solutions causes a change in the configuration of the exocyclic double bonds, with the formation of photoisomers [1,2]. Oxidative degradation occurs simultaneously, producing biliverdin as an intermediate and causing decomposition of the tetrapyrrole skeleton, with the formation of products that do not absorb visible light and are more easily excreted than bilirubin [3]. These products were identified by Lightner *et al.* [4] in the urine of irradiated newborns. The two processes play a role in the phototherapy and thus are intensively studied. It is clear from the literature that the excretion of bilirubin degradation products and the mechanism of the photochemical process are still not quite defined under *in vivo* conditions. The present paper makes a contribution to a solution of this problem.

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The photooxidation of bilirubin is catalysed by various dyes that function as energy carriers [5]. The catalytic effect of riboflavin was first described by Sanvordecker and Kostenbauder [6]. We continued to study this process and found out that, in addition to sensitization, a direct photochemical reaction occurs in which riboflavin and bilirubin act as an electron acceptor and donor, respectively, with biliverdin as a product [7,8]. Another photoproduct, lumirubin, was recently isolated [1,9,10], and some authors consider it to be the main metabolite of bilirubin excreted during phototherapy.

This paper describes a study of the mechanism of photodegradation of bilirubin, with special emphasis on the role of riboflavin. This mechanism was described by a set of differential equations, which were solved. The kinetic parameters were quantitated using special programs. Riboflavin, as a vitamin, is a normal component of the blood and it can be shown that its catalytic effect cannot be neglected during the study of bilirubin metabolism.

EXPERIMENTAL

Chemicals

Bilirubin was obtained from Merck (Darmstadt, Germany). Biliverdin was prepared by the oxidation of bilirubin by ferric chloride in an acetic acid medium [11]: $E_{660\ nm}$ = 15 100 (methanol). Riboflavin-5-phosphate was obtained from Hoffmann La Roche (Basel, Switzerland). 2,4-Dimethyl-3-carboxymethylpyrrole (relative molecular mass, M = 137; $C_8H_{11}ON$). The standard monopyrrole derivative was synthesized in the Research Institute for Pharmacy and Biochemistry (Prague, Czechoslovakia).

Acetonitrile and N,N-dimethylformamide were from Merck, human serum albumin (HSA) from Imuna (Michalany, Czechoslovakia) and adenosine, reduced glutathione, cysteine and 4-aminopyrazolone from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

To prepare the bilirubin sample, 1.75 mg of the substance was weighed, 0.2 ml of 0.1 M KOH was added and the solution formed was diluted to 10 ml with distilled water (the concentration was 300 μM). This stock solution was appropriately diluted after the addition of 1.2 equivalents of HSA, and the pH was adjusted to 6.8 with a phosphate buffer. The concentration of the riboflavin-5-phosphate was 1 μM.

The irradiated samples were deproteinated with acetonitrile–methanol (1:1, v/v), containing 1% of 1 M NH_4Cl; 1.5 ml of the mixture were added to 0.5 ml of the sample. The solution was allowed to stand in darkness for 15 min, centrifuged (3000 g) and injected into the column. The solutions were protected against light by wrapping in aluminium foil.

The serum samples should be treated as soon as possible, to prevent secondary

reactions occurring in the blood; otherwise, the serum must be frozen at −20°C and α-tocopherol possibly added as a stabilizing agent [8]. The proteins are removed as described above.

Column liquid chromatography

The concentrations of the bilirubin photoisomer and biliverdin were determined by liquid chromatography (LC) on a Pye Unicam PU 4020 instrument with a Spherisorb ODS column (Pye Unicam, Cambridge, U.K.; 100 mm × 4.6 mm I.D., particle size 5 μm) using photometric detection at 395 nm. The absorption coefficients of the photoisomers and biliverdin are identical at this wavelength and under the given experimental conditions, so that quantitative analysis is possible (ε = 34 400), using biliverdin as the standard and correcting for the photoisomers according to Malhorta and Ennever [12].

The mobile phase used for both the model samples and the serum contained a succinic acid–Tris buffer (pH 5.8) in methanol–acetonitrile–dimethylformamide (60:12:2, v/v): 10 ml of the buffer were added to 74 ml of the mixture and diluted with distilled water to 100 ml. The flow-rate was 1 ml/min, and the temperature 21–24°C.

The degradation products were determined in the model sample using a mobile phase of an orthophosphoric acid–Tris buffer (pH 4.0) in aqueous acetonitrile. A 30-ml volume of acetonitrile was added to 10 ml of the buffer with a concentration of 0.1 *M*, and the mixture was diluted with distilled water to 100 ml. In this case, a wavelength of 280 nm was used for the detection.

The bilirubin concentration was determined spectrophotometrically on a Unicam SP 800 instrument at 455 nm; the molar absorption coefficient ε of the complex with HSA was 48 000.

Irradiation

The solutions were irradiated in 0.5–1.0 cm quartz cuvettes closed with PTFE stoppers, using a Medicolux B lamp that is employed in phototherapy. The radiant power in the region 400–500 nm is 5 $\mu W/cm^2$ at a distance of 80 cm.

Mathematical model and its computer implementation

The mathematical model for bilirubin degradation is based on the scheme shown in Fig. 1. It consists of a set of three ordinary linear differential equations with constant coefficients:

$$\frac{dx_1}{dt} = -k_1x_1 + k_2x_2$$

$$\frac{dx_2}{dt} = -(k_2 + k_4 + k_5)x_2 + k_1x_1$$

$$\frac{dx_3}{dt} = -k_3x_3 + k_4x_2$$

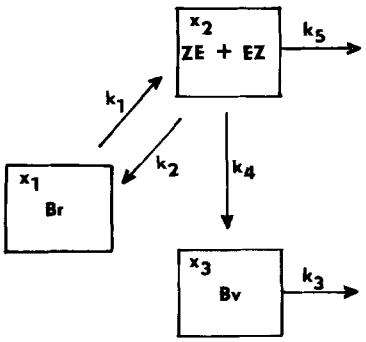

Fig. 1. Reaction scheme for photodegradation of the bilirubin complex with human serum albumin in the presence of riboflavin. Abbreviations: Br = bilirubin; Bv = biliverdin; x = molar concentrations; ZE + EZ = photoisomers; k = the relative rate constant.

This model was developed on a PC/AT computer, and its parameters were dynamically optimized using special software based on Nelder–Meade methods.

The sum of the squares of the deviations between the experimental values and the model output served as the criterion for the estimate of the optimal parameter values. The results of the optimization procedure confirmed that the model is adequate.

RESULTS

The photoisomers recorded in the LC experiments were identified on the basis of the time dependence of the increase of the intensity of the peaks and the decrease following addition of phosphoric acid, which causes rapid conversion into the stable ZZ isomer. The ZE and EZ isomers could be differentiated because of their different behaviour in the presence of human and bovine serum albumin. The formation of the ZE isomer predominates in the presence of human albumin, whereas both the isomers are formed in the presence of bovine albumin [1,2]. After removal of the light source, reversible conversion into the stable isomer takes place with a half-life of 190 min at 37°C. Lumirubin was isolated by thin-layer chromatography on silica gel [1]. The reproducibility of determination, expressed in terms of the coefficient of variation, is 5% for spectrophotometry, 10% for biliverdin and 15% for the photoisomers, using liquid chromatography.

Fig. 2 depicts the degradation of bilirubin by light in the presence and the absence of riboflavin. The comparison indicates that the catalyst causes the half-life of the degradation to decrease by a factor of 4.5, and so this effect at a concentration typical for blood is not negligible. Table I lists numerical data for the degradation products formed and the consumption of oxygen.

Fig. 3 shows a typical chromatogram obtained in an experiment that modelled the photochemical processes, with an initial bilirubin concentration of 150 μM; the formation of the photoisomers ZE and EZ, biliverdin and lumirubin is clearly visible.

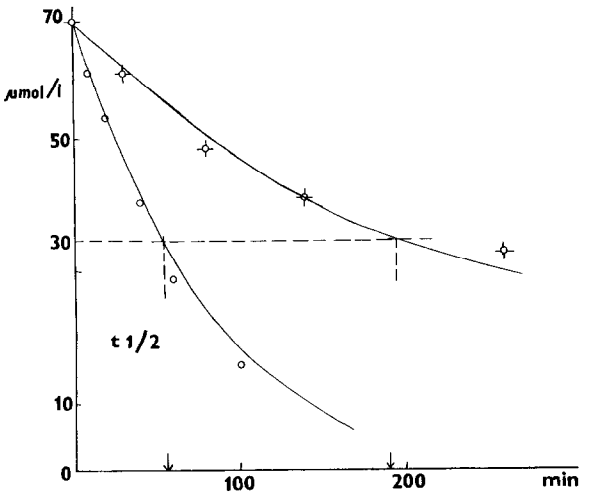

Fig. 2. Bilirubin photodegradation in the presence of FMN at a concentration of 1 μM (pH 6.7). The initial bilirubin concentration was 70 μM in a 0.5-cm cuvette. (⊕) without the catalyst; (○) with the catalyst.

TABLE I

PHOTODEGRADATION OF THE BILIRUBIN COMPLEX WITH HUMAN ALBUMIN, IN THE PRESENCE AND ABSENCE OF THE CATALYST

Values are in μM. Br = bilirubin; Bv = biliverdin; L = lumirubin; ZE + EZ = photoisomers; P = degradation products expressed in terms of μM loss of bilirubin.

Time (min)	Br	Bv	EZ + ZE	L	P	Oxygen pressure (mmHg)
With FMN						
0	71	0.2	1.0	–	–	177
5	65	2.1	12.8	–	3.9	
15	56	6.4	12.0	0.5	8.6	
35	40	16.0	8.6	1.4	15.0	
70	20	20.3	3.0	3.0	31.0	165 (70)
Without FMN						
0	58	0.2	0.8	–	–	180
10	56	0.8	17.2	–	–	
30	54	1.2	12.8	0.8	1.8	
60	49	1.6	10.0	1.2	6.4	
120	42	2.7	5.7	1.5	10.3	
180	38	4.2	3.4	1.2	15.0	173 (40)

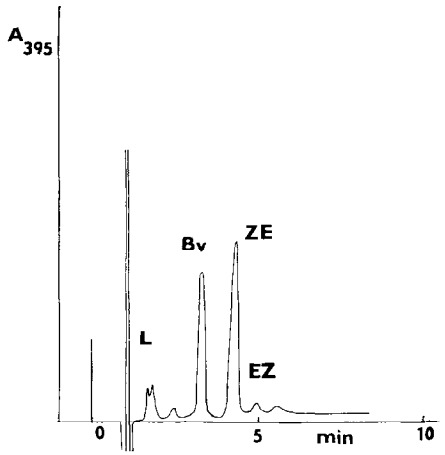

Fig. 3. Chromatogram of the products of degradation of bilirubin on irradiation in a model experiment: concentration, 150 μM in a 0.5-cm cuvette; irradiation time, 25 min. The concentrations of biliverdin (Bv), *ZE* + *EZ* photoisomers and lumirubin (L) were 20.5, 36.0, 1.7 and 5 μM, respectively.

Model experiments with HSA-containing solutions were evaluated using the appropriate differential equations. The experimental points and the calculated curves are given in Fig. 4, representing the degradation of the *ZZ* isomer and the formation of the photoisomers and biliverdin as a function of time. The formation of lumirubin is included in the formation of the isomers. The experimental and calculated values show good agreement.

It follows from these results that, according to the relative rate constants, the initial process is rapid photoisomerization, followed by photooxidation with the formation of biliverdin as an intermediate.

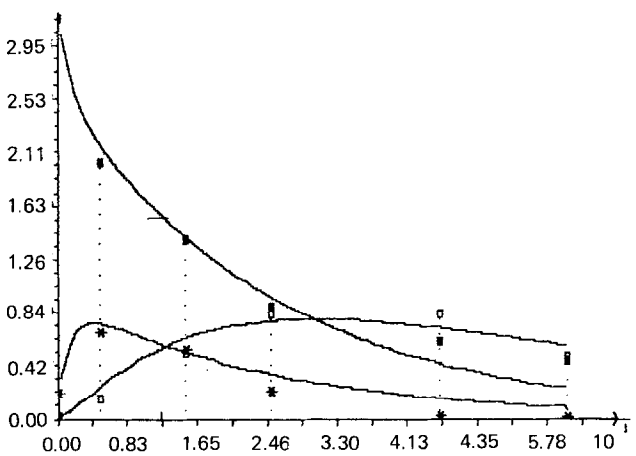

Fig. 4. The solution of the mechanism of bilirubin–HSA complex photodegradation using the mathematical model. The curves represent the calculated values, and the indivual points were obtained experimentally: (■) degradation of the *ZZ* isomer; (□) formation of biliverdin; (*) photoisomer formation. Initial concentration of bilirubin, 31.6 μM; concentration of FMN, 1 μM (pH 6.8); *x*-axis, min; *y*-axis, μM.

Table II gives numerical values for the dependence on the catalyst concentration (1 and 3 μM).

The effect of oxygen is important for the photochemical degradation of bilirubin. Under anaerobic conditions, the catalytic reaction stops when equilibrium is reached, and the conversion of riboflavin (R) into the leuco form (RH_2), which is catalytically inactive [7,8]:

$$Br + R \rightleftharpoons Bv + RH_2$$

The oxygen consumption is substantially larger than required by the stoichiometry of the biliverdin formation, which indicates that photooxidation occurs by the following steps. (The oxygen consumption was calculated from the partial pressures).

The formation of the final degradation products in the stage of solution discoloration was followed in model experiments. This process is manifested by a decrease in the absorbance at 455 nm and an increase in the absorbance in the region 250–300 nm, whereas the products of degradation of the tetrapyrrole skeleton absorb radiation.

TABLE II

DEPENDENCE OF THE RATE OF PHOTOOXIDATION AND PHOTOISOMERIZATION OF BILIRUBIN *ZZ* ON THE CATALYST CONCENTRATION UNDER AEROBIC CONDITION IN A PHOSPHATE BUFFER (pH 6.8)

Time (min)	Br*ZZ*[a] (μM)	*ZE* + *EZ* (μM)	Bv (μM)	P (μM)
FMN concentration 1 μM				
0	31.6	–	0.2	–
5	20.2	6.8	1.5	3.1
15	14.1	5.4	5.1	7.0
25	8.9	2.1	8.3	12.5
45	6.0	0.2	8.0	17.1
60	4.5	–	4.9	22.3
Relative rate constants (μmol/min): $k_1 = 0.150$; $k_2 = 0.305$; $k_3 = 0.029$; $k_4 = 0.079$; $k_5 = 0.671$. (see Fig. 1).				
FMN concentration 3 μM				
0	34.5	–	0.6	–
3	22.8	4.2	2.2	4.7
8	11.0	2.0	7.4	14.5
15	7.8	0.2	1.1	25.3
30	5.2	–	–	27.9
Relative rate constants (μmol/min): $k_1 = 0.178$; $k_2 = 0.317$; $k_3 = 0.243$; $k_4 = 0.430$; $k_5 = 0.387$ (see Fig. 1).				

[a] Br*ZZ* = stable isomer of bilirubin: other abbreviations as in Table I.

Fig. 5 shows a chromatogram representing the formation of the degradation products of bilirubin after irradiation for 30 min in a Petri dish.

To test the effects of various biochemically important substances on the mechanism of bilirubin degradation, the influence of uric and salicylic acid, α-tocopherol, adenosine, glutathione, cytochrome C, potassium iodide, etc. was studied: the results are summarized in Table III.

In order to compare the results of the model experiments with those obtained *in vivo*, the formation of the bilirubin metabolites was followed during phototherapy. Fig. 6 gives a chromatogram obtained in the first day of the phototherapy.

The chromatogram demonstrates that rapid formation of the photoisomers occurs during the first hours of irradiation, whereas the formation of biliverdin is strongly suppressed. The formation of lumirubin is also very low. An orientative determination of the degradation products in the serum was also carried out. Using detection at 285 nm, the presence of degradation products in the serum was demonstrated; the dipyrrole derivative predominated, its concentration being *ca.* 2.4 mg per 100 ml of serum.

DISCUSSION

The study of the model of bilirubin photodegradation has demonstrated that riboflavin strongly affects the mechanism, both quantitatively and qualitatively, even at concentrations typical for *in vivo* conditions. The formation of biliverdin as an intermediate has been clearly proved. The catalyst affects not only the photooxidation, but also the photoisomerization. This was indicated by a com-

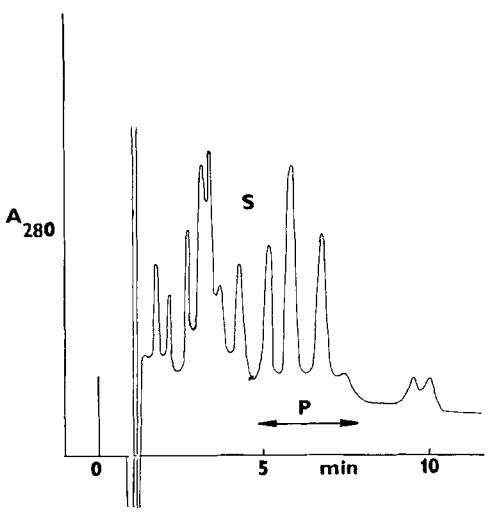

Fig. 5. Chromatogram of the degradation products of the tetrapyrrole skeleton of bilirubin after 30 min irradiation. Initial concentration of bilirubin, 150 μM; buffer pH, 8.3; irradiation in a Petri dish; S = standard monpyrrole derivative; P = dipyrrole derivatives.

TABLE III

EFFECT OF SOME INHIBITORS AND STABILIZING AGENTS ON THE PHOTODEGRADATION OF BILIRUBIN UNDER AEROBIC CONDITIONS

The state after 15 min irradiation by a 5 $\mu W/cm^2$ source in the wavelength range 400–500 nm, with the stabilizing agent at a concentration of 10^{-3} *M*; FMN concentration, 1 μM. Abbreviations as in Table I.

Substance added	Initial concentration (μM)	$t_{1/2}$ (min)	Br (μM)	Bv (μM)	*ZE* + *EZ* (μM)	P (μM)
Oxygen	71	38	56	6.4	12.8	8.6
Uric acid	71	55	59	3.2	3.5	8.8
3-Phenyl-2,3-dimethyl-4-amino-pyrazolone	66	65	56	3.0	1.0	7.0
α-Tocopherol	74	90	66	3.0	6.0	5.0
Cysteine	63	39	52	6.0	8.9	5.0
Reduced glutathione	60	35	47	5.0	8.0	8.0
Adenosine	67	34	48	5.0	5.0	14.0
Salicylic acid	67	37	52	4.0	7.0	11.0
Potassium iodide	71	130	62	1.3	3.0	6.3
Cytochrome c	62	105	54	5.5	0.0	2.5

parison of the rate constants and their dependence on the concentration of the catalyst, and from an experiment carried out under anaerobic conditions in which bilirubin degradation was stopped and the photoisomerization process slowed down [7,8]. Migliorani *et al.* [13] observed a similar effect when they applied laser

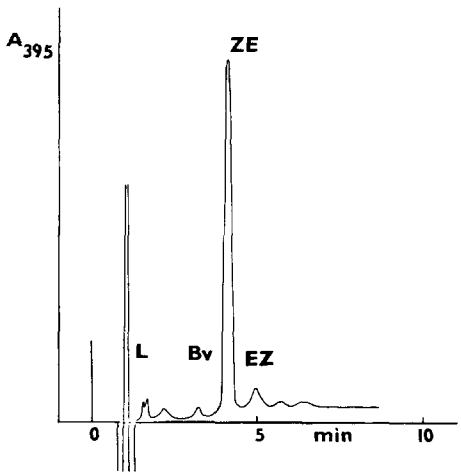

Fig. 6. Chromatogram of the blood serum of a newborn suffering from hyperbilirubinemia, during the first day of irradiation. The concentration of biliverdin (Bv), the *ZE* + *EZ* photoisomers and lumirubin (L) were 0.4, 41.5, 1.5 and 0.5 μM, respectively.

irradiation to the photochemical degradation of bilirubin. The reaction mechanism *in vivo* is different, as far as biliverdin formation is concerned. Biliverdin concentrations in the blood of irradiated newborns are sometimes lower than the detection limit of the method. We assume that this deviation is caused by action of enzyme systems that strongly affect the formation of this intermediate [14].

The effect of some inhibitors and stabilizers under physiological conditions can be explained as follows. Cytochrome c and potassium iodide are typical inhibitors of the photochemical process. Glutathione and cysteine stabilize the catalyst against the effect of light, as shown elsewhere for the photooxidation of various amino acids [15]. The protective effect of these substances in the irradiation of DNA solutions was pointed out by Korycska-Dahl and Richardson [16] and Wilson and Wardman [17]. Salicylic acid displaces bilirubin from the complex bond to proteins. The purine derivative adenosine, which is a component of nucleic acids, does not participate in the photochemical reaction under the given conditions. In agreement with this, we did not observe any changes in the spectrum after a 3-h irradiation of an RNA solution in the presence of a physiological concentration of riboflavin. Speck and Rosenkrantz [18] observed a change in the structure of the RNA spectrum, but under completely different, non-physiological conditions, at riboflavin concentrations higher by several orders of magnitude.

The application of the mathematical model and its computer treatment have brought new findings. It follows from the verified scheme that the initial reaction is rapid photoisomerization, followed by photooxidation with biliverdin intermediate. The rate constants obtained give information on the roles of the individual stages in the reaction scheme. The photodegradation has been demonstrated by us both in model experiments and *in vivo*, and the mechanism is in agreement with the work of Callahan *et al.* [19] and Schmid and Hammaker [20], who followed the degradation of labelled bilirubin in blood during phototherapy and found that degradation to non-toxic and more easily excretable products occurs in the blood during the first hours of irradiation. It is also in agreement with the work of Lightner *et al.* [4], who demonstrated the presence of these products in het urine of irradiated newborns. The kinetics of formation of the photoisomers in the absence of riboflavin were mathematically treated by Itoh and Onishi [21]. We have obtained analogous results in studying the reversible formation of the photoisomers.

The experimental results indicate the significance of riboflavin for the photochemical process of bilirubin degradation *in vitro* and *in vivo*. The question arises of the occurrence of hypovitaminosis during phototherapy, and the possibility of supplementing the vitamins required. Our results also open new possibilities of the use of the stabilizing effect of some of the substances tested. The problem of vitamin supplementing is often discussed in the literature with a view to improving the phototherapy [22–24]. We have verified successful supplementing with physiological doses of riboflavin.

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