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STUDY OF THE MECHANISM OF THE PHOTOCHEMICAL OXIDATION OF BILIRUBIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The photochemical degradation of bilirubin was studied in vitro using high-performance liquid chromatography and spectrophotometry. Attention was centred on the formation of biliverdin, which is produced as an intermediate in the photooxidation catalysed by riboflavin. Approximate values of the overall and partial relative rate constants were calculated using the physicochemical criteria for a pseudo-monomolecular reaction. A more precise evaluation was made by non-linear regression programmes on a Hewlett-Packard 9835 A computer. In addition to the formation of biliverdin, attention was also paid to accompanying processes affecting the reaction mechanism, which were explained as aggregation and dimerization of bilirubin and biliverdin. It was also found that during phototherapy of newborn babies suffering from hyperbilirubinaemia the level of biliverdin in the plasma increased. The results are discussed from the point of view of phototherapy.

INTRODUCTION

The photochemical degradation of bilirubin continues to be of interest as this process is the basis of the phototherapy of hyperbilirubinaemia of newborn babies and the mechanism has not yet been fully clarified. In the degradation of bilirubin by light, two main principles are involved: (a) photoisomerization, considered at present to be the most efficient, consisting of a reversible change in the configuration on the exocyclic double bonds in bilirubin [1,2], and (b) photooxidation, in which bilirubin is oxidized or its molecules are oxidatively dissociated.

The photooxidation of bilirubin is catalysed by various dyes acting as energy carriers [3]. The photochemical degradation of bilirubin in the presence of riboflavin has been investigated by Sanvordecker and Kostenbauder [4]. We have studied the reaction in greater detail, as a model system for phototherapy, as riboflavin is always present in the organism [5–7]. We found that the reaction is reversible and the riboflavin acts as the acceptor and bilirubin as the donor of

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electrons. The reaction is pseudo-monomolecular and the main product is biliverdin [8].

Biliverdin is an intermediate in haem degradation, depending on an enzyme system of haem oxygenase, cytochrome C reductase and biliverdin reductase. The products involve bilirubin, biliverdin and various fragments of the tetrapyrrole skeleton [9–11]. Recently another enzyme, bilirubin oxidase, has been identified; it oxidizes bilirubin in the presence of oxygen to biliverdin and is probably bound to mitochondria [12,13]. In this work we continued with the investigation of the kinetics of the degradation of bilirubin by light, using high-performance liquid chromatography (HPLC) in order to follow the side and subsequent reactions. A mathematical model was processed on a Hewlett-Packard 9835 A computer in order to assess the model parameters by non-linear regression methods.

EXPERIMENTAL

Apparatus and analytical conditions

The contents of bilirubin and biliverdin in model experiments were determined by HPLC and spectrophotometry. The experimental conditions are described elsewhere [8]. The light source was a Medicolux lamp with a radiant power of 5.8 $\mu W/cm^2 \cdot nm$ in the range 400–500 nm and at a distance of 80 cm. The solutions were irradiated in Petri dishes or in closed quartz cuvettes with a layer thickness of 0.5 cm. A Spherisorb ODS (5 μm) column (100 mm $\times$ 4.6 mm I.D.) was used. Biliverdin was detected at 375 nm and IX-α-biliverdin was used as the standard [8]. The mobile phase consisted of 80% methanol in 0.01 *M* Tris buffer of pH 3.5–5.8. The oxygen pressure was measured with an Astrup instrument.

Procedure

In determination of biliverdin in model samples, the secondary reactions were eliminated, as they had an adverse effect on the precision of the measurements (especially the oxidation of bilirubin by atmospheric oxygen). The following procedure yielded the best results: to 0.5 ml of an irradiated solution of bilirubin with albumin were added 1.3 ml of methanol, 0.2 ml of 20% sodium chloride solution and 1 ml of dimethylformamide, the solution was purged with pure nitrogen for 5 min and the clear solution after centrifugation was injected into the column. The plasma samples were treated immediately, if possible. When the determination was performed one day after the sample collection, the blood was centrifuged and the plasma was acidified with citric acid and stored in a refrigerator at $-20°C$. Further treatment was the same as above, but methanol acidified with hydrochloric acid was used for denaturation, a stabilizer was added to the mixture and extraction into 2 ml of chloroform was carried out in a nitrogen atmosphere. As stabilizers, salicylic acid, α-tocopherol and 4-amidopyrazolone, which according to Tanaka and Muroa [13], is an efficient inhibitor of bilirubin oxidase, were tested. We obtained good results using 10^{-3} *M* α-tocopherol. The chloroform extract was evaporated in vacuo and the residue was dissolved in the mobile phase and injected on to the column.

The study of the mechanism of bilirubin photooxidation was based on the ex-

perimental determination of the loss of bilirubin with time, using spectrophotometry at 460 nm ($\epsilon = 56\,000$ l/mol·min). The biliverdin concentration was determined by HPLC with photometric detection at 375 nm. The reaction mechanism was expressed in terms of a simple model, whose scheme and mathematical description are given in Fig. 1.

The model consists of a set of side and subsequent reactions and the basis is the determination of the rate of disappearance of bilirubin with time (constant k), monitoring of the formation and degradation of biliverdin using HPLC and the solution of the appropriate differential eqns. 2 and 3:

$$k = \frac{2.303}{t} \cdot \log\left(\frac{a}{a-x}\right) \tag{1}$$

where $k = k_1 + k_2$ and a = initial concentration of bilirubin.

$$\frac{dx}{dt} = -(k_1 + k_2)x \qquad x(0) = x_0 \tag{2}$$

$$\frac{dy}{dt} = k_1 x - k_3 y \tag{3}$$

$$x(t) = x_0 \exp[-(k_1 + k_2)t]$$

$$y(t) = A\exp[-(k_1 + k_2)t] - \exp(-k_3 t)$$

$$A = k_1 x_0 / (k_3 - k_1 - k_2)$$

Approximate values of the rate constants k_1, k_2 and k_3 were calculated according to the criteria for side and subsequent reactions [14]. As a criterion of correctness, the equation was used that expresses the dependence of the rate of formation and degradation of biliverdin with time:

$$t_{max} = \frac{1}{k_3 - k_1} \cdot \ln\left(\frac{k_3}{k_1}\right) \tag{4}$$

Parameters k_1, k_2 and k_3 were calculated by non-linear regression with a regression criterion of the number of squares of the differences between the experimental values and the model output.

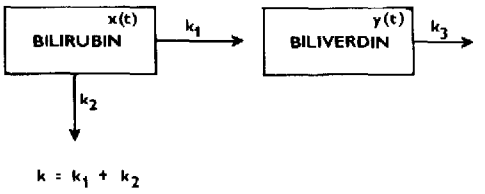


Fig. 1. Simplified model of bilirubin photooxidation, which forms the basis for the calculation of the relative rate constants of the formation and degradation of biliverdin.

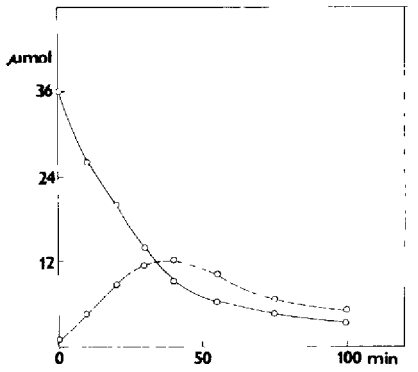
RESULTS

During irradiation of a bilirubin solution, the intensity of the orange coloration decreases and a green coloration due to biliverdin appears. In the last stage of irradiation, a pink coloration appears, which we attribute to the aggregation of biliverdin.

Fig. 2 shows the photochemical degradation of bilirubin in a medium of bovine albumin and the formation of biliverdin. It can be seen that the biliverdin formation exhibits a maximum after 38 min.

Fig. 3 depicts the HPLC determination of biliverdin. The analysis of the differences between the experimental values and the model output helped in elucidating the reaction mechanism. The applicability of the model was tested statistically in each experimental situation by comparison of the variance of the method of measurement and the variance of the residues, using the F-test. Examples of the application of the computational techniques to the mechanism studied are given in Figs. 4 and 5, which demonstrate the correlation of the experimental values with the calculated curve corresponding to the formation and degradation of biliverdin by light. In the first instance a discharge lamp used in phototherapy was employed and in the other sunlight was applied.

Values of the relative rate constants are given in Table I, expressing the dependences on the experimental conditions (pH, radiation intensity and wavelength). The determination of the kinetic parameters of the studied reaction indicates that the formation of biliverdin corresponds, depending on the experimental conditions, to 25–75% of the initial concentration of bilirubin. It can be seen in Fig. 4 that the experimental curve deviates slightly from the calculated curve, espe-



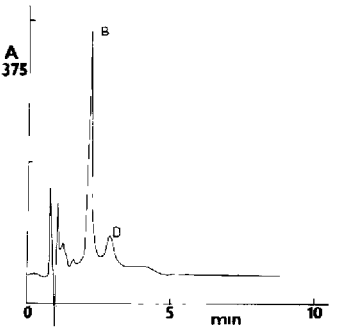


Fig. 2. Graphical representation of the degradation of bilirubin and the formation of biliverdin after irradiation in an open Petri dish in a phosphate buffer of pH 6.7. Initial bilirubin concentration, 37 µmol/l; initial riboflavin phosphate concentration, 10 µmol/l; light source, phototherapeutic discharge lamp.

Fig. 3. Chromatogram for the determination of biliverdin in experiment No. 3. Flow-rate, 1.0 ml/min; mobile phase, Tris buffer (pH 5.8) + 80% methanol. Concentration of 0.01 M. After 20 min irradiation.

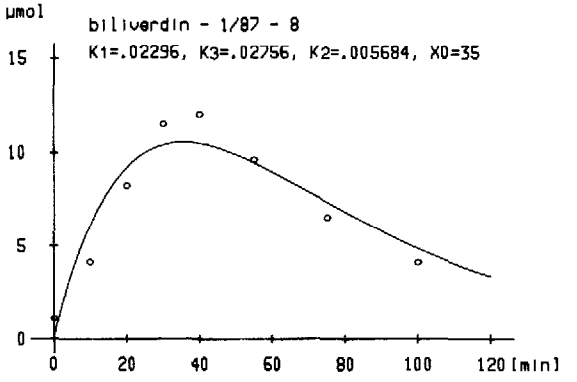


Fig. 4. Experimental and calculated curves for biliverdin formation in experiment No. 3. ○, Experimental points; solid line, calculated curve. Relative rate constants: $k=0.02865$; $k_1=0.0230$; $k_3=0.02756$; $k=k_1+k_2$.

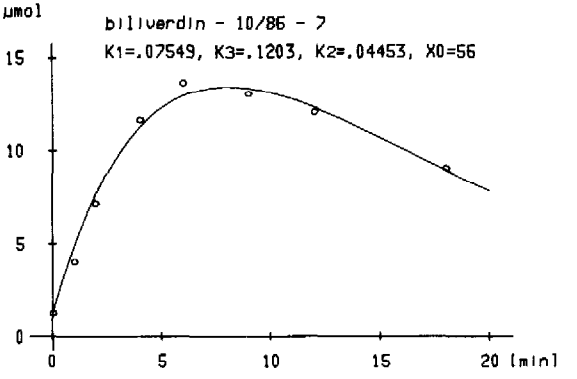


Fig. 5. Experimental and calculated curves for biliverdin formation in a buffer of pH 6.8. Concentration of bilirubin with albumin, 56 µmol/l; concentration of riboflavin phosphate, 5 µmol/l; irradiated in a 0.5-cm cuvette with sunlight. Rate constants: $k=0.12002$; $k_1=0.07549$; $k_3=0.1203$; $k=k_1+k_2$.

cially on the right-hand side. This effect can be explained by the aggregation or dimerization of biliverdin.

Aggregation and dimerization of biliverdin is manifested by a pink coloration. The product can be extracted into diethyl ether after acidification with acetic acid, evaporated, dissolved in methanol and an absorption spectrum obtained with maxima at 550–560 and 330 nm. On acidification of the solution with phosphoric acid and recording of the spectrum after 20 min, the absorbance changes, indicating a reversible transformation of the pink product into biliverdin. On irradiation of a bilirubin solution in a phosphate buffer of pH 8.25 (without albumin) and monitoring of the time dependence by HPLC, another peak appears, corresponding to the dimer of biliverdin. The ratio of the two peak areas is reversible: on removal of the source the biliverdin peak increases at the expense of the dimer peak and on addition of phosphoric acid the dimer peak disappears completely and the content of biliverdin increases. The same effect has been observed in monitoring the photolysis of biliverdin under the same conditions, which demonstrates that the photoproduct formed is a derivative of biliverdin. Isolation

TABLE I

CALCULATED RELATIVE RATE CONSTANTS FOR THE DEGRADATION OF BILIRUBIN, THE FORMATION OF BILIVERDIN AND ITS PHOTOLYSIS AS A FUNCTION OF THE MEDIUM, LIGHT INTENSITY AND THE CONCENTRATION OF RIBOFLAVIN PHOSPHATE

Experiment No.*	Medium	pH	Concentration (μmol/l)		$t_{1/2}$ (min)	k (μmol/min)	k_1 (μmol/min)	k_3 (μmol/min)
			Bilirubin	Riboflavin				
1	Petri dish, closed	6.5	55	20	41	0 0161	0.0119	0.0150
2	Petri dish, closed	6.5	55	10	63	0 0109	0.0082	0.0106
3	Petri dish, open	6.4	36	10	24	0.0286	0.0230	0.0276
4	Petri dish, open	6.5	36	1	115	0.0053	0.0016	0.0036
5	Cuvette, closed	6.6	55	15	31	0.0196	0.0142	0.0232
6	Cuvette, closed	6.6	54	10	45	0.0128	0.0089	0.0125
7	Cuvette, closed, sunlight	6.8	56	10	4.5	0.1200	0.0754	0 1203

*Light intensity: experiments Nos. 1–6, 5.8 μW/cm²·nm; No. 7, 24.0 μW/cm²·nm.

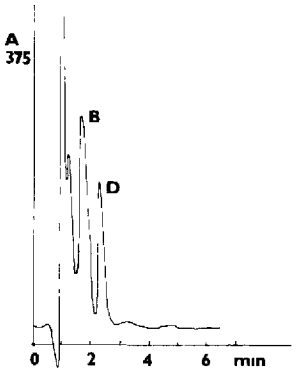


Fig. 6. Chromatogram of an irradiated solution of bilirubin in phosphate buffer of pH 8.25. Concentration of bilirubin, 39 μmol/l; concentration of riboflavin, 5 μmol/l; flow-rate after 20 min irradiation, 1.0 ml/min; mobile phase, Tris buffer of pH 6.0 + 80% methanol. The chromatogram shows the formation of biliverdin dimer.

of the dimer is difficult and is complicated by the fact that the dimer is continuously in equilibrium with the monomer and its presence can only be demonstrated under conditions optimal for the equilibrium state.

The formation of the dimer is depicted in Fig. 6. The monomer–dimer (reversible aggregate) equilibrium is shifted by light in favour of the dimer.

The consumption of oxygen during the photooxidation of bilirubin with albu-

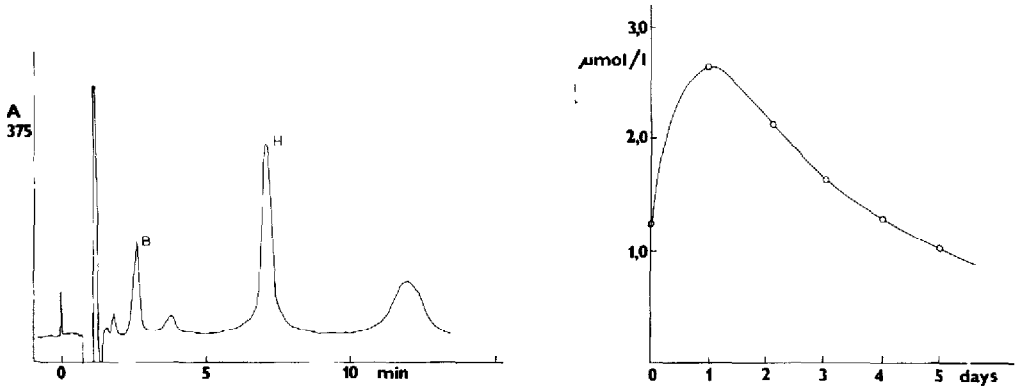


Fig. 7. Chromatogram for the determination of biliverdin in the plasma of a newborn baby suffering from hyperbilirubinaemia and treated by phototherapy. B = bilirubin IX-α isomer; H = Fe protoporphyrin. Elution with Tris buffer (pH 5).

Fig. 8. Graph representing the formation of biliverdin during phototherapy of a newborn baby suffering from hyperbilirubinaemia. Abscissa, time in days; ordinate, biliverdin concentration in μmol/l.

min was monitored in a closed quartz cuvette. The consumption of oxygen was measured by following the pressure on an Astrup instrument. It was found that on degradation of 31 μmol of bilirubin, 18 μmol of biliverdin are formed and 25.8 μmol of oxygen are consumed (corrected for the blank), 9 μmol corresponding to the formation of biliverdin and 18 μmol to a further oxidation.

The effect of the light intensity is demonstrated in the experiment with the sunlight of intensity 24.0 $\mu W/cm^2 \cdot nm$ in the range 400–500 nm. The irradiation was carried out in a phosphate buffer of pH 6.8 in a closed cuvette. The calculated curve for biliverdin formation is in very good agreement with the experimental values, as can be seen in Fig. 5, which shows that the rate of degradation and the formation of biliverdin are several times higher than those with the use of the phototherapy source.

To find approximately the extent to which this mechanism is operative during phototherapy, several experiments were carried out by monitoring biliverdin, as the primary oxidation product, in the plasma of irradiated newborn babies. The results of the HPLC determination are given in Fig. 7.

The dependence of the biliverdin concentration on the irradiation time is shown in Fig. 8. It can be seen that on the first and second days of irradiation the biliverdin concentration increases perceptibly in the plasma of a newborn baby. Fractions of a micromole of biliverdin were even found in the urine during this period. We also attempted to demonstrate the formation of biliverdin glucuronides in the plasma of newborn babies suffering from hyperbilirubinaemia, using the HPLC method recommended by Li et al. [15] for the determination of bilirubin glucuronide and based on methanolysis and conversion into mono- and dimethyl esters. We have demonstrated the presence of glucuronides in the plasma by this procedure.

DISCUSSION

The study of the mechanism of bilirubin degradation by light and of photoisomerization has been possible by the use of HPLC. We studied the mechanism of the process in the presence of riboflavin, which catalyses the reaction and is permanently present in tissues as a vitamin. We have demonstrated experimentally that it acts as a catalyst even at concentrations around 1 μmol, typical of physiological conditions.

A comparison of the experimental results with the calculated values led to the discovery of aggregation and dimerization, which occur as side processes during bilirubin photolysis. Dimerization of bilirubin was first pointed out by Brodersen [16]. Dimerization and aggregation of biliverdin were described by Haidel and Kraus [17] and Braslavsky et al. [18]. Dimerization of the biliverdin derivative ethylbiliindone by light in an oxidizing medium was described by Scheer and Kraus [19] and Schoch et al. [20], who also used HPLC for the identification with a similar column to ours. They observed a similar behaviour of the dimer in the presence of the monomer (the absorption spectrum in methanol exhibited maxima at 570 and 325 nm). In a study of the binding of bilirubin and biliverdin to proteins, Giralt et al. [21] studied the reversible polymerization of bilirubin and biliverdin adsorbed on various resins. The polymers obtained could be converted into the original substances. Henning et al. [22] described the selective adsorption of biliverdin on resins containing arginine and lysine as terminal groups. Knobloch and Miler [23] observed, in a study of the detoxification of bilirubin by human granulocytes and leucocytes, an adsorption effect that could be expressed by an isotherm describing the decrease in the bilirubin concentration.

Schmidt and Hammaker [24] pointed out an alternative role of oxidation in the phototherapy of bilirubinaemia and used labelled bilirubin. Bruss et al. [25] described an increase in the concentrations of bilirubin and biliverdin in the blood of experimental animals after an infusion of bilirubin; the concentration of biliverdin increased up to 60-fold. Hirota et al. [26] and Gonzales et al. [27] described an increase in the concentration of biliverdin in the urine of rabbits to whom biliverdin was administered. Kutty and Maines [28] obtained further evidence on the metabolic function of biliverdin; they assumed that biliverdin has an important function as the regulator of haem oxygenase and thus affects the whole metabolism of haem degradation.

It can be concluded from our results that the phototherapy involves a significant effect of photooxidation, represented by the formation and separation of biliverdin, followed by degradation of the tetrapyrrole skeleton, which is also in agreement with the work of Lightner et al. [10]. This is also important for other reasons. We consider photoisomerization to be the principal process in degradation of bilirubin, as the reaction is very rapid and thus apparently precedes the photooxidation. It is necessary to consider the experimental findings of Lamola et al. [29], who demonstrated that during phototherapy a maximum of 15% of the total bilirubin content is converted into photoisomers. This state is caused by the establishment of equilibrium, as the formation of photoisomers is a re-

versible process. This equilibrium is disturbed by the formation and separation of the less toxic and easier metabolizable intermediate biliverdin.

The results indicate that, in addition to photoisomerization, a complex detoxification process must be taken into consideration during phototherapy, including photooxidation, the role of enzymes and physicochemical factors, such as dimerization and adsorption.

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