FUNCTION OF FLAVINS IN PHOTOLYSIS OF BILIRUBIN *in vitro*

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Received March 26th, 1980

In the course of photochemical oxidation of bilirubin, riboflavin works as a reversible electron acceptor undergoing a transformation into the riboflavin-leucoform. Simultaneously bilirubin is irreversibly decomposed. Under anaerobic conditions the bilirubin–riboflavin system behaves as an electrochemical equilibrium system, characterized by its redox potential. Leucoform formation was detected by spectrophotometry, polarography and potentiometry. Under aerobic conditions the expected photochemical equilibrium is not established probably owing to the reoxidation of riboflavin-leucoform by oxygen. Of the flavin derivatives present in blood riboflavin, FAD and FMN were tested, riboflavin being the most effective one. This paper summarizes the model studies of biochemical function of flavins, which could be applied to the phototherapy of neonatal jaundice.

Much attention has been devoted to the bilirubin photochemistry in view of its importance in the therapy of neonatal jaundice[1,2]. Bilirubin is degraded by light under aerobic as well as under anaerobic conditions[3,5]. According to model experiments in various organic solvents it is supposed that under aerobic conditions photooxidation is mediated by the singlet oxygen[4]. In recent literature[6–8] the mechanism of this process has been vividly discussed with regard to the possibility of application under *in vivo* conditions[6–8]. Although the contribution of the anaerobic bilirubin degradation to the process is, according to Ostrow[3], also important, less attention has been devoted to it up to now. Following bilirubin photodegradation products were identified: methylvinylmaleinimid, hematic acid, 5,5'-diformyl-dipyrrolmethan and other fragments of the tetrapyrrol skeleton[4,5,9,10]. Some authors assume that biliverdin is formed in the course of photolysis as an intermediate[9,11]. Bilirubin is present in blood mostly in the *IX* form, α-isomer[11], bound to albumin[2,12]. On irradiation bilirubin reacts with some substrates under formation of photoaddition products[13]. The photolysis of bilirubin is accelerated by some dyes, for instance by methylene blue or riboflavin.

Already Cremer and coworkers[14] considered possible therapeutic utilization of riboflavin as a sensibilizator in the phototherapy of neonatal jaundice. Sanvordeker and Kostenbauder[15] were concerned with photodegradation in the presence of riboflavin under conditions *in vitro*. They explain the degradation of bilirubin by a singlet oxygen mechanism, by the energy transfer from light excited riboflavin to the oxygen molecule. They consider the contribution of an anaerobic process as negligible. A stimulated degradation of bilirubin in the presence of FMN *in vivo* using Gunn rats was already reported[16]. Hodr and coworkers[17] reported studies of the effect of radiation on the system bilirubin–riboflavin–albumin using a special discharge lamp developed for the phototherapy of neonatal jaundice. In addition to riboflavin, the effect of other vitamins of the B group was studied and was found negligible. Meisel and Jähring[18] studied the photodegradation of bilirubin in the presence of FMN and methylene blue. The oxygen consumption was detected manometrically.

Recently a series of examples of photoreduction of flavins in the presence of suitable electron donors as various nitrogen containing compounds like EDTA, methionine, sarcosine, cysteine *etc.* has been reported[19-21]. In this reaction a reversible riboflavin reduction and an oxidative degradation of the substrate takes place. We have shown that bilirubin can also work as an electron donor in this reaction and a short preliminary report dealing with this phenomenon was submitted[22]. This paper reports the results of the studies of the effect of flavins on bilirubin photolysis using a suitably selected model and is a contribution to the theoretical knowledge needed for the phototherapy of neonatal jaundice.

EXPERIMENTAL

Material

Bilirubin was a product of Merck, FRG ($A_{1cm}^{1\%} = 1\,030$ at 460 nm in chloroform), 1 mg was dissolved in 0·2 ml of 0·1M-KOH and distilled water (bubbled by N_2) was added up to 10 ml. A standard lyophylized preparation of bilirubin with albumin from Lachema, ČSSR (0·8 mg bilirubin + 80 mg albumin) was dissolved in 4 ml of distilled water, $c = 342$ μmol/l. Riboflavin from Spofa, ČSSR, was crystallized from diluted acetic acid and dried at 100 °C. To 10 mg of riboflavin + 2 ml 98% acetic acid distilled water was added up to 100 ml, $c = 263$ μmol/l. FMN was obtained from Hoffmann LaRoche, FAD from BDH, England. Phosphate buffers pH 6, 6·5, 7·3 and 8·5, concentration 0·16 mol/l were used. Pure nitrogen was used for removing oxygen.

Methods

For spectrophotometric measurements a spectrophotometer Unicam SP-800 was used, fluorescence spectra were measured using a spectrofluorimeter Opton M-4. Quartz cells with teflon or glass stoppers were used. Polarographic curves were registered using a polarograph MP 160. Potentiometric measurements were performed with a potentiometer from Laboratorní přístroje, Prague, using a platinum electrode as a measuring one and a standard calomel electrode (S.C.E.) as reference, in a special device constructed for working in inert atmosphere. As a light source a discharge lamp modified by addition of indium was used. The intensity of radiation of such a lamp in the wavelength range 450—460 nm is one order of magnitude higher than in the other spectral regions within the range 350—580 nm (ref.[23]). This lamp is applied in our Institute to the phototherapy of neonatal jaundice. The energy of this light source measured at a distance of 80 cm by an actinometric method using Fe^3 oxalate (ref.[24]) was $E = 1{\cdot}4 \,.\, 10^4$ erg cm^2 s^{-1}.

In anaerobic experiments the oxygen was removed in a special vessel by bubbling the buffered solution by pure nitrogen passed through an alcaline pyrogallol solution. Bilirubin was added after oxygen removal by a syringe in a stream of nitrogen. The solution was transfered to a cell filled by nitrogen, tightly closed by a teflon stopper and irradiated from a distance of 80 cm. The course of photodegradation was followed by spectrophotometry or potentiometry. The process of oxygen elimination was controlled polarographically and it was found that under experimental conditions mentioned above the oxygen concentration in the bubbled solution decreases to about 10^{-6} mol/l, which was also confirmed by a control of the partial pressure of oxygen using an Astrup apparatus. The formation of the leucoform of riboflavin, the reduction character of which excludes any presence of oxygen, represents an indirect evidence that anaerobic conditions have

been reached. Negligible traces of oxygen are removed during the first minute after irradiation was started by reoxidation of the riboflavin leucoform. In similar studies of photoreduction of FMN in the presence of EDTA (using helium) Fife and Moore[25] made the same experience. The vessels were protected from light by an aluminium foil.

RESULTS

Anaerobic and Aerobic Bilirubin Degradation

It is obvious from the comparison of anaerobic and aerobic degradation of bilirubin by light in the presence of riboflavin (Fig. 1*a*, *b*) that under anaerobic conditions (*b*) a characteristic retardation of the photochemical reaction takes place whereas under aerobic conditions (*a*) the process proceeds with a practically unchanged reaction rate. The difference in the reaction rates is graphically expressed in Fig. 2 as the dependence of absorbance at 460 nm on time. From the graph it is obvious that the rate of the absorbance decrease plotted in logarithmic scale is a linear function of time. The photodegradation thus occurs as a pseudo-monomolecular reaction. Under anaerobic conditions some retardation becomes evident already after 10 minutes, but the photolysis never stops completely. The residual rate is determined by the slope, which corresponds to the degradation of bilirubin alone by light. The slope deter-

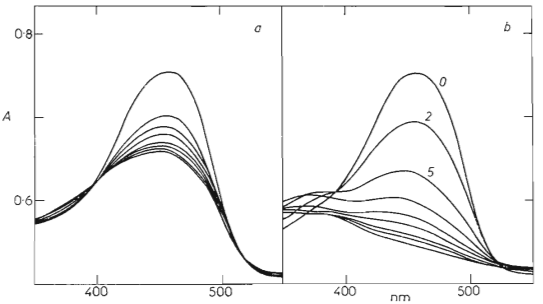

FIG. 1

Absorption spectra of the mixture of bilirubin, riboflavin and albumin in a phosphate buffer, pH 6·5, as a function of time of irradiation under aerobic (*a*) an anaerobic (*b*) conditions. Concentration of bilirubin and riboflavin 25 μmol/l, concentration of albumin 15 μmol/l. Time intervals 0, 2, 5, 10, 20, 30, 40, 50 and 65 min. Irradiated in a quartz cell, 1 cm layer

mining the rate of the aerobic process is equal to the tangent to the curve obtained under anaerobic conditions. Thus we can say that initial rates of both reactions are approximately the same. The relative rate constant k' calculated from the slope has a value $1{\cdot}15 \,.\, 10^{-1}$ min^{-1}.

Formation of the Riboflavin Leucoform

The formation of the riboflavin-leucoform under anaerobic conditions was proved by polarography, potentiometry and spectrophotometry. Preliminary studies of the system under anaerobic conditions were made by polarography. A fully reversible polarographic wave was obtained with riboflavin[21]. After 10 minutes of irradiation of the mixture containing 50 μmol/l riboflavin and 25 μmol/l bilirubin in a buffer, pH 7·3, partial decrease of the riboflavin wave below the zero line of the galvanometer occurred, indicating a reversible reduction. After bubbling the solution by air the wave returns to its original position.

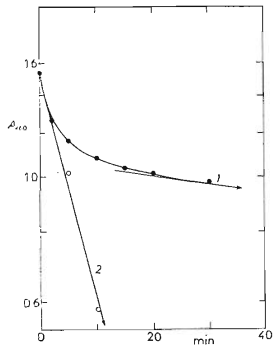

FIG. 2

Absorbance at 460 nm as a function of time in the preceding experiment. *x*, time in min, *y*, absorbance in logarithmic scale. Conditions: *1* anaerobic, *2* aerobic

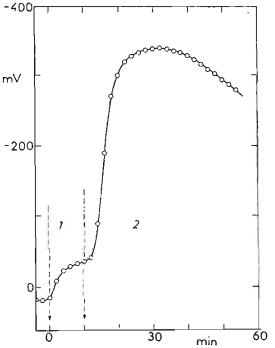

FIG. 3

Potential as a function of time of irradiation of the system riboflavin–bilirubin–albumin in a phosphate buffer, pH 7·3, under anaerobic conditions. Concentration of bilirubin and riboflavin 25 μmol/l, concentration of albumin 15 μmol/l, volume of irradiated solution 25 ml, layer 3 cm. *x*, time in min, *y*, potential in mV against S.C.E. *1* dark, *2* light

The observed photoreduction of riboflavin assumes an electrochemical process characterized by a change in the potential. The potential of the system bilirubin–riboflavin–albumin in a buffer, pH 7·3, under anaerobic conditions was measured (Fig. 3). After bubbling separately the solution of bilirubin and riboflavin in the dark and after mixing, a small shift of the potential occurs. After irradiation an intensive shift of the potential from region 0 to the values around −0·340 V against S.C.E. takes place. We believe that this shift is due to riboflavin photoreduction. The potential is kept at this value for several minutes, afterwards it begins to decrease slowly, which we believe to be due to a parallel effect of riboflavin photolysis at the expense of the photoreduction. However, the contribution of photolysis to the overall process is small. The photolysis is also accompanied by a change of the potential, but the course is different and the characteristic equilibrium is not established. This experiment proves that the reaction of riboflavin with bilirubin under anaerobic conditions is an electrochemical process, which to a small extent takes place also in the dark.

The leucoform of riboflavin does not absorb in the visible region of the spectrum and does not reveal the intensive yellow-green fluorescence of riboflavin. Based on these phenomenons are also the spectrophotometric and fluorimetric tests of the leucoform. After riboflavin photoreduction by bilirubin under anaerobic conditions followed by bubbling with air a characteristic increase of the absorbance at 447 nm (absorption maximum of riboflavin) and an increase of fluorescence intensity at 520 nm occurs in agreement with the leucoform reoxidation. From these values the concentration of riboflavin leucoform and the stoichiometric ratio of reacting substances (indicating a bimolecular reaction) can be calculated rather exactly.

Photolysis of Riboflavin as a Competing Reaction

In the case of photochemical degradation of bilirubin in the presence of riboflavin the photolysis of riboflavin itself cannot be excluded. The contribution of photolysis to the reaction under study was determined by comparing the rate of bilirubin degradation based on photoreduction with the rate of riboflavin photolysis in a buffer, pH 7·3. Bilirubin concentration was determined from the absorbance at 460 nm and the photolytic riboflavin degradation was determined from the characteristic fluorescence at 520 nm. Riboflavin photolysis goes about 4 times slower than bilirubin destruction (Fig. 4).

Characterization of the Photochemical Reaction

The photochemical character of the reaction follows from the linear dependence of the reaction rates of both substances on the amount of energy absorbed at the wavelength 460 nm (Fig. 5). An approximate value of the quantum yield θ was calculated

from the equation derived by Hatchard and Parker[24], which gives the number of molecules reacting with one photon. A value of θ equal 0·0074 results from the equation mentioned above after substituting the measured and calculated values. The value of θ was also checked indirectly by comparing the reaction rates of a known system riboflavin–EDTA, where θ equal 0·06 was found[26]. For the system riboflavin–bilirubin $\theta = 0{\cdot}01$ was obtained.

The characteristic retardation of the photochemical reaction under anaerobic conditions can be explained as an effect of a reversible reaction between the products of the photochemical process and the original substances. The reaction can be expressed by a general scheme:

$$\frac{c(\mathrm{RH}) \,.\, c(\mathrm{B})}{c(\mathrm{R}) \,.\, c(\mathrm{BV})} = \frac{k_1}{k_2} = K\,, \qquad (I)$$

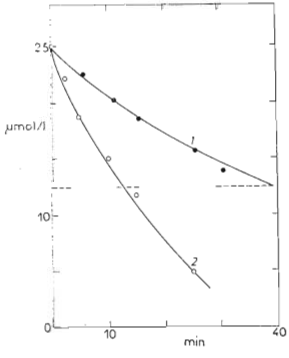

FIG. 4

Degradation of riboflavin and bilirubin after irradiation in a phosphate buffer, pH 7·3, under aerobic conditions. Layer of solution 1 cm, concentration of bilirubin and riboflavin 25 μmol/l, concentration of albumin 15 μmol/l. Concentration of riboflavin was measured from the intensity of fluorescence at 520 nm (*1*), that of bilirubin from the absorbance at 460 nm (*2*). *x*, time in min, *y*, concentration in μmol/l

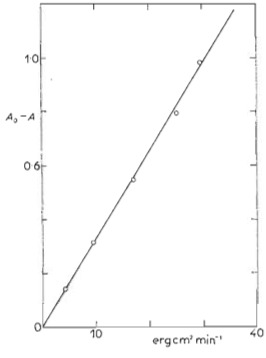

FIG. 5

Photodegradation of the mixture bilirubin–riboflavin–albumin as a function of the amount of energy absorbed at 460 nm, in a phosphate buffer, pH 7·3. Concentration of riboflavin 12·5 μmol/l, concentration of bilirubin 25 μmol/l. Irradiated in a layer 1 cm. *x*, energy absorbed in erg $cm^2\ min^{-1}$, *y*, absorbance difference $A_0 - A$ at 460 nm, $A_0 = 1{\cdot}5$

where $c(RH)$ and $c(R)$ are the concentrations of riboflavin–leucoform and of riboflavin, respectively, after attaining the equilibrium; $c(B)$ and $c(BH)$ are the concentrations of bilirubin and of an unknown reaction product.

The concentration of riboflavin leucoform at equilibrium was calculated from the absorbance increase at the wavelength 447 nm after aerating the solution. The concentration of bilirubin was calculated from the corrected absorbance at 460 nm. The calculation was based on the experiment at pH 7·3, concentrations of both substances being 25 μmol/l, time 15 minutes. By substitution into equation 1 an approximate value of the constant $K = 0{\cdot}35$ is obtained, the rate constant k_1 can be calculated from the slope of the reaction rate under aerobic conditions $k_1 = 1{\cdot}1 \cdot 10^{-1}\ \text{min}^{-1}$.

Comparison of the Effect of Riboflavin, FMN *and* FAD

In view of the biochemical importance a comparison of the catalytic effects of riboflavin derivatives occurring in blood, namely of riboflavin, FMN and FAD (Table I) was carried out. The measurements were made using solutions containing 12·5 μmol/l of flavins and 25 μmol/l of bilirubin with albumin, in a buffer, pH 7·3. From the comparison of the half-time values it follows that the electron transfer activity of FAD is 5 times lower than that of riboflavin.

Effect of Albumin and Inhibition

The effect of albumin, which was added in order to increase the solubility of bilirubin and also to approach as well as possible the *in vivo* conditions, results in higher stability of bilirubin, as was already observed by other authors[3]. Photochemical degradation of bilirubin in a phosphate buffer, pH 8·3, occurs twice faster in absence of albumin than in its presence.

TABLE I
Comparison of catalytic effects of riboflavin derivatives

Substance	Half-time, min	$k' \cdot 10^{-3}\ \text{min}^{-1}$
Bilirubin	195	3·5
Bilirubin–riboflavin	14	49·5
Bilirubin FMN	19	36·4
Bilirubin FAD	65	10·6

The reaction mechanism of photolysis is inhibited by KI and by pyrogallol in concentrations 20 μmol/l, a small increase of the reaction rate was observed in the presence of cysteine and glutathione.

DISCUSSION

According to the experimental results, riboflavin in bilirubin photooxidation under anaerobic conditions works as a reversible electron acceptor. This finding is in accordance with the function of riboflavin as coenzyme in various natural redox systems and with its photoreduction during photooxidation of some amino acids. It agrees also with the observation concerning the involvement of an anaerobic process in the photolysis of bilirubin[3]. However, our findings are not in accordance with another paper[15], where the involvement of an anaerobic process in bilirubin degradation in the presence of riboflavin is considered as negligible and the whole mechanism is attributed to the effect of singlet oxygen.

We assume that the main process occurring during photochemical degradation of bilirubin in the presence of riboflavin is the transfer of an electron to the triplet state of riboflavin, similarly as in the case of riboflavin photoreduction in the presence of some amino acids. This assumption also agrees with the inhibition by KI.

It can thus be concluded that bilirubin photooxidation in the presence of riboflavin is a complicated photochemical reaction originating in the redox character of riboflavin, which is also responsible for the electrochemical properties of the system. The kinetics of the process is a function of the light energy absorbed by the system. The characteristic retardation of the reaction occurring under anaerobic conditions is explained by the involvement of a reversible reaction between the products and the original substances. Because various successive and competing reactions interfere, the reaction is never fully suppressed. On the basis of the spectral characteristics of the intermediates we assume that the primary product of the photochemical oxidation of bilirubin is biliverdin. Finally the bilirubin is irreversibly degraded to unknown products.

Isomerisation of bilirubin by light[27,28], leading to an equilibrium state under anaerobic conditions, was described recently. Polar, better soluble products are formed during this isomerisation which are excreted as metabolites. It is probable that this fast process is also a preceeding reaction in our experiments but it in is comparison with photooxidation smaller. According to the present knowledge, bilirubin is eliminated by normal photolysis and isomerisation during phototherapy. We assume that the photooxidation described by us and based on the oxidation-reduction function of flavine in blood also participates in this proces.

Under aerobic conditions the expected photochemical equilibrium is never established because of the interference due to reoxidation of riboflavin leucoform by oxygen. Participation of the singlet oxygen cannot be excluded. However, taking

into account the fact that the reoxidation of riboflavin-leucoform occurs also in the dark, it is probably immaterial and in our new approach not necessary for the interpretation of the whole mechanism.

REFERENCES

1. Luccy J. F.: Semin. Hemat. *9*, 127 (1972).
2. Bohrman R. F., Brown A. K., Currie E. R.: J. Pediatr. *84*, 135 (1974).
3. Ostrow J. D., Branham R. V.: Gastroenterol. *58*, 15 (1970).
4. Donagh A. F.: Biochem. Biophys. Res. Comm. *44*, 1306 (1971).
5. Lightner D. A., Cu A.: Life Sci. *20*, 723 (1977).
6. Kurtin W. E.: Photochem. Photobiol. *27*, 503 (1978).
7. Lightner D. A.: Photochem. Photobiol. *26*, 427 (1977).
8. Matheson J. B. C., Curry N. U., Lee J.: J. Amer. Chem. Soc. *96*, 3348 (1974).
9. Ostrow J. D., Nicholson D. C., Stoll N. S.: Gastroenterol. *60*, 196 (1971).
10. Gray C. H., Kulczycka, Nicholson D. C.: J. Chem. Soc., Perkin Trans. *1*, 288 (1972).
11. Bonnett R., Davis J. F., Hursthowe M. B.: Nature (London) *262*, 326 (1976).
12. Pedersen A. O., Schonheyder F., Brodersen R.: Eur. J. Biochem. *72*, 213 (1977).
13. Manitto P., Monti D.: Experientia *26*, 379 (1972).
14. Cremer R. J., Perryman P. W., Richards D. H.: Lancet *1*, 1094 (1958).
15. Sandvordeker D. R., Kostenbauder H. B.: J. Pharm. Sci. *63*, 404 (1974).
16. Kostenbauder H. B., Sanvordeker D. R.: Experientia *29*, 282 (1973).
17. Hodr R., Janda J., Houdková V.: Čs. Pediatr. *32*, 78 (1977)).
18. Meisel P., Jähring D.: Acta Biol. Med. Germ. B *37*, 1229 (1978).
19. Merkel V., Nickerson W. J.: Biochim. Biophys. Acta *14*, 303 (1954).
20. Friesell W. R., Chung C. W., Mackenzie C. G.: J. Biol. Chem. *234*, 1295 (1959).
21. Knobloch E.: Methods Enzymol. 18 B, 358. Academic Press, London 1971.
22. Knobloch E., Hodr R., Herzman J., Houdková V.: Acta Vitaminol. Enzymol. *3*, 182 (1981).
23. Baley Z., Dvořáček V., Mathesius V.: Tesla Electronic *8*, 112 (1975).
24. Hatchard C. G., Parker C. A.: Proc. Roy. Soc. Ser. A *235*, 518 (1956).
25. Fife D. J., Moore W. A.: Photochem. Photobiol. *29*, 43 (1979).
26. Radda G. K., Calwer M.: Biochemistry *3*, 384 (1964).
27. Stoll M. S., Zenone E., Ostrow J. D.: Biochem. J. *183*, 139 (1979).
28. Donagh Mc A., Lightner B., Wooldridge F.: Chem. Soc. Commun. *110*, 112 (1979).

Translated by J. Šponar.