CONVERSION OF BILIRUBIN BY TRANSFER OF ELECTRONIC EXCITATION ENERGY GENERATED IN THE PRESENCE OF HUMAN METHEMOGLOBIN

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The conversion of bilirubin into colourless products has been induced by transfer of electronic excitation energy and followed spectrophotometrically. The triplet excitation energy was generated by oxidation of 2-methylpropanal with oxygen in solution under catalytic action of human methemoglobin. A slower kinetics of transformation of bilirubin via this "photochemical reaction without light" has been observed for the bilirubin bound to human albumin as compared with the bilirubin alone.

For a number of years bilirubin has been object of intensive photochemical research[1]. The reason is that the processes taking place during the generally used phototherapy of neonatal jaundice have not yet been sufficiently investigated. The mentioned phototherapy was demonstrated for the first time by Cremer et al.[2] who showed that the yellow colour (due to bilirubin) of newborn babies suffering from jaundice disappears on irradiation with blue light.

The experiments in vitro carried out with the plasma of newborn babies showed that the yellow colour disappears on irradiation. The rate of this phototransformation was increased in the presence of photosensitizing dyestuffs[3]. A review by McDonagh[4] gives a number of possible reaction paths of transformation of bilirubin involving photoisomerization, sensitized photooxidation with singlet oxygen, and photooxidation with participation of radicals. Lightner et al.[5] showed that the irradiation of bilirubin causes its rapid photoisomerization into products of much better solubility in water as compared with the original non-irradiated bilirubin. These processes are supposed by Lightner to be the main ones in the phototherapy.

Matheson et al.[6] studied photooxidation of bilirubin in various solvents and with various concentrations of oxygen and bilirubin. The results interpreted as a reaction of singlet oxygen show that the quantum yield of bilirubin in triplet state is 0·05 to 0·08. The quantum yield of the same reaction at the concentrations typical of a living organism is estimated at 0·01.

Sloper and Truscott[7] dealt with the excited states of bilirubin itself generated by laser flash-photolysis. The quantum yields found for formation of triplet bilirubin

in aqueous solutions were below 0·05. The authors discuss the importance of the results obtained for the phototherapy of neonatal jaundice and they also suggest a scheme of photodegradation of bilirubin in the presence of albumin.

From the survey of literature it follows that the excited triplet state of bilirubin is important for its phototransformation. However, the photoexcitation produces the triplet state of bilirubin in very low yields due perhaps to a little efficient intersystem crossing[6]. It is known[8] that oxidation of 2-methylpropanal with oxygen present in aqueous solution and with the catalysis by horse-radish peroxidase (HRP) produces the excited acetone in its triplet state. The transfer of this triplet energy to a suitable acceptor can be utilized for inducing a photochemical reaction often called "photochemistry without light"[9] or "photochemistry without excitation radiation"[10]. We decided to find whether or not it is possible to carry out the phototransformation of bilirubin by transfer of the triplet excitation energy generated enzymatically. For the enzyme we chose a substance of animal origin – human methemoglobin, which in this case operates as an oxidase[11].

EXPERIMENTAL

The bilirubin was a product of fa. Serva (Heidelberg), the human serumalbumin was a product of fa. Imuna n.p. (Šarišské Michaľany) which was rid of salts on a Sephadex G-25 column before use. The human methemoglobin was prepared by oxidation of a solution of hemoglobin with potassium hexacyanoferrate(III) and subsequent gel filtration of products on a Sephadex G-25 column[11].

2-Methylpropanal was prepared by oxidation of 2-methyl-1-propanol with a dichromate–sulfuric acid mixture according to a method by Lipp[12].

The chemiluminescence of triplet acetone was measured by means of a detection apparatus of liquid scintillation counter ISOCAP (Nuclear, Chicago). The spectrophotometric measurements were carried out with a Specord UV-VIS spectrophotometer (Zeiss, Jena) in the region of 30 000 to 12 000 cm^{-1}. The generation of triplet state of acetone was realized in the Sörensen phosphate buffer pH 7·0 containing methemoglobin of the concentration of $1 \cdot 10^{-6}$ mol dm^{-3}, EDTA $4{\cdot}95 \cdot 10^{-4}$ mol dm^{-3}, and ethanol 0·025 mol dm^{-3}. For investigation of transfer of excitation energy, bilirubin was added to the above-mentioned solution to make the final concentration of $2{\cdot}5 \cdot 10^{-5}$ mol dm^{-3}, and the generation of triplet state of acetone was initiated by addition of 2-methylpropanal to the final concentration of 0·026 mol dm^{-3}. In the cases of investigation of transfer of triplet energy to the bilirubin–albumin complex, albumin solution was added to the reaction mixture beside bilirubin so that the stoichiometric ratio of the bilirubin–albumin complex formed might be 1 : 1.

RESULTS

The electronically excited acetone in the triplet state was generated by the oxidation of 2-methylpropanal catalyzed with human methemoglobin acting as a dioxygenase[11]. The time dependence of chemiluminescence intensity of the system given is presented in Fig. 1. The system was used as a source of electronic excitation energy

for the photochemical transformation of bilirubin which was followed by the differential spectrophotometry. Figure 2 presents, as an example, the differential spectra of bilirubin solution in a reaction mixture with triplet acetone recorded at several time intervals from the beginning of the reaction. As the reference solution we adopted bilirubin in buffer without the system generating the triplet acetone. Beside the absorbance difference corresponding to bilirubin (ΔA_{450}) also that corresponding to the Soret band of methemoglobin (ΔA_{415}) can be seen in Fig. 2.

The time dependence of the absorbance difference at $\lambda = 450$ nm caused by the transfer of triplet excitation energy to bilirubin alone and to bilirubin–albumin complex is represented in Fig. 3. For comparison, Fig. 4 presents the effect of transfer of excitation energy on the absorbance of the Soret band of methemoglobin both in the presence and in the absence of serumalbumin.

DISCUSSION

Generation of electronically excited states of biologically important substances without application of the excitation radiation is extensively studied, however, mainly with the use of a plant enzyme – the horse-radish peroxidase[13]. There exists

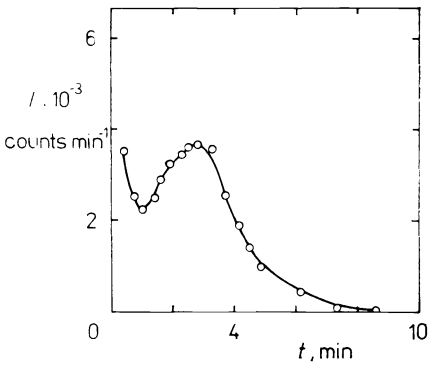

FIG. 1

Time course of chemiluminescence (I) of the triplet acetone generated by oxidation of 2-methylpropanal with oxygen with catalytic action of methemoglobin

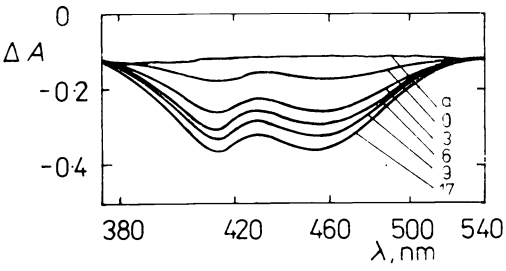

FIG. 2

Differential absorption spectra of the bilirubin present in the system methemoglobin–2-methylpropanal–oxygen. a The zero line recorded in the case when the cell contained no 2-methylpropanal, 0—17 the spectra recorded after 0, 3, 6, 9, and 17 min from the addition of 2-methylpropanal. Concentrations: bilirubin $2{\cdot}5 \cdot 10^{-5}$ mol dm^{-3}, methemoglobin $1 \cdot 10^{-6}$ mol dm^{-3}, 2-methylpropanal 0·026 mol dm^{-3}; phosphate buffer pH 7·0

a presumption that photochemical reactions "without light" can take place in living organisms, both in plants and animals[9]. For the purpose of our study of a reaction from an electronically excited state enzymatically generated and possible conclusions of physiological nature it was necessary to find and investigate a catalytic protein of animal origin. We chose human methemoglobin which has both peroxidase and oxidase enzyme effects[11]. With catalytic action of human methemoglobin as an oxidase we succeeded in generating electronically excited triplet acetone by oxidation of 2-methylpropanal (Fig. 1). The transfer of the excitation energy thus generated onto bilirubin resulted in a transformation of the latter substance manifested by an absorbance decrease (Fig. 2) and having obviously photochemical character. Bilirubin in a complex with human albumin[14] was found to be more resistant to the photochemical transformation (Fig. 3). The photochemical transformation of bilirubin in the presence of albumin proceeded within the whole period it was spectrophotometrically followed (Fig. 3). However, the electronic excitation energy generated during the experiment was also transferred to the enzyme (methemoglobin) itself and led to spectral changes manifested in absorbance decrease of the Soret band. As it can be seen from Fig. 4 the considerable decrease of absorbance of the Soret band of methemoglobin alone resembled in its course that of bilirubin alone (Fig. 3). On the other hand, in the presence of albumin a (not very distinct) absorbance decrease of methemoglobin took place only at the beginning of reaction, being not further changed during its further course (Fig. 4).

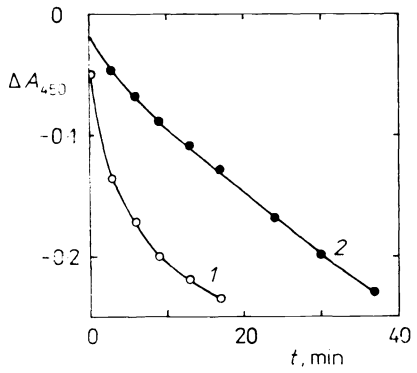

FIG. 3

Time course of differential absorbance (ΔA_{450}) of bilirubin alone (*1*) and of bilirubin–albumin complex (*2*). Concentrations: bilirubin $2{\cdot}5 \cdot 10^{-5}$ mol dm^{-3}, albumin $2{\cdot}8 \cdot 10^{-5}$ mol dm^{-3}; phosphate buffer pH 7·0

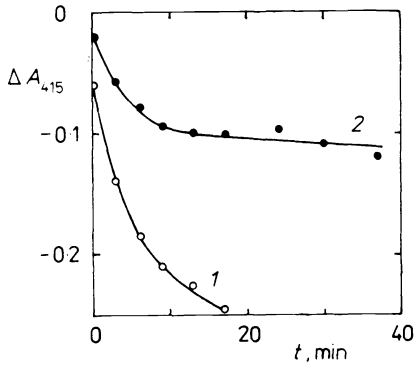

FIG. 4

Time course of the absorbance difference of the Soret band (ΔA_{415}) of methemoglobin alone (*1*) and methemoglobin in the presence of albumin (*2*). Concentrations: methemoglobin $1 \cdot 10^{-6}$ mol dm^{-3}, albumin $2{\cdot}8 \cdot 10^{-5}$ mol dm^{-3}; phosphate buffer pH 7·0

For elucidation of the photochemical transformation of bilirubin during the phototherapy Matheson et al.[6] suggest a mechanism starting from the first singlet state of bilirubin. This state, according to the mechanism suggested, at first is transformed to the conformous singlet state and then, by the intersystem crossing (ISC) induced by oxygen, it leads to formation of triplet-state bilirubin. The latter species on returning to the ground electronic state generates the singlet oxygen ($^1\Delta_g$) which is, obviously at least in part, responsible for the therapeutic process.

Photoisomerization of bilirubin from its triplet state formed by intersystem crossing of the irradiated bilirubin–albumin complex is presumed by Sloper and Truscott[7] on the basis of their own studies. Both the paper by Matheson et al.[6] and that by Sloper and Truscott[7] indicate the key role of triplet-state bilirubin in the phototherapy of neonatal jaundice. The results of our studies, where the triplet state was obtained directly by a transfer of triplet excitation energy, confirm the role of triplet-state bilirubin in its photochemical transformation. There arises a question whether the given way of photochemical transformation of bilirubin without application of excitation radiation can play a role in natural degradation of bilirubin in the organism of a newborn baby during jaundice. In order to answer this question, we would have to find a substance of aldehydic nature naturally occurring in human organism whose oxidation catalyzed with methemoglobin would result in formation of excited triplet products. Another possibility at hand is to try to find a relatively harmless substance of aldehydic nature and use it to induce the photochemical transformation of bilirubin in organism without application of any excitation radiation.

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