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The Photochemistry of Bilirubin

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The present state of research into the photochemical reactions of bilirubin in various media is examined. Data are presented on certain physical, chemical, and physicochemical properties of bilirubin. Attention is specially concentrated on photodynamic decomposition processes and photoisomerisation of bilirubin. The data are discussed in connection with the urgent problem of the use of light in the treatment of hyperbilirubinemia in children.
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I. INTRODUCTION

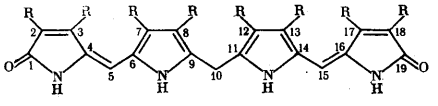
The yellow pigment bilirubin (BR) is formed in the organism mainly by the degradation of haemoglobin released into the blood plasma from decomposing erythrocytes. In contrast to the protein and "free" iron, which are also haemoglobin degradation products, BR is not utilised by the organism and furthermore can have a toxic effect on the latter. Therefore the removal of BR from the organism is an important physiological process, which, however, is to a large extent complicated by the insolubility of BR in aqueous media so that BR is transported in the organism by being bound in water-soluble complexes and as a result of the formation of water-soluble derivatives. In particular, BR circulates in the blood channels in a complex with serum albumin (SA) and with participation of liver cells is removed to the common bile ducts mainly in the form of esters with one or two glucuronic acid molecules. When the system for the removal of BR from the organism breaks down, a pathological state—hyperbilirubinemia—can arise; this state is accompanied by the deposition of BR in the skin, which acquires a yellow hue as a result. This factor is the basis for the common term "jaundice" used in medical practice.

Recently the treatment of neonatal jaundice by irradiating the skin with visible light in a specific wavelength range has come to be widely employed. The successful application of phototherapy led investigators to undertake studies on various aspects of the photochemistry of BR. This review is in fact devoted to this problem. Bearing in mind that in recent years there have been no review communications on BR in Soviet literature we also considered topics necessary for the understanding of the photochemistry of BR, which provide an idea about the most important physicochemical, physical, and chemical properties of BR.

II. THE STRUCTURE AND PROPERTIES OF BILIRUBIN

1. Structure

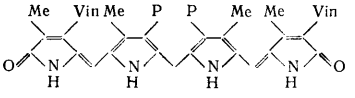
Bilirubin constitutes a set of structural isomers of a tetrapyrrole compound having the general formula



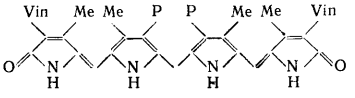
(1)

where R = methyl (Me), vinyl (Vin), or carboxyethyl (P = propionic acid residue) under conditions such that in all instances there are two vinyl, two carboxyethyl, and four methyl groups.

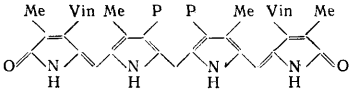
It has come to be assumed that only one of the structural isomers of BR (the so called BR IXα) is formed in the organism:



(IXα), (2)



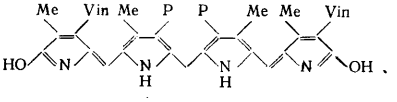
(IIIα), (3)



(XIIIα). (4)

This isomer has come to be referred to simply as bilirubin in medical, biological, and chemical practice. However, there are data showing that the blood serum of the newborn suffering from jaundice contains only 62–70% of BR IXα, while the remaining BR consists of the isomers IIIα (10–19%) and XIIIα (16–20%).[1]

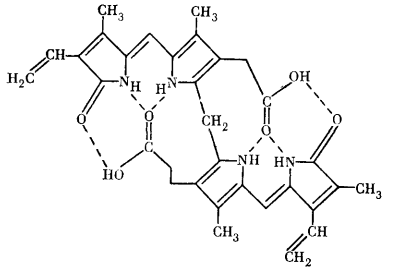
The bislactam or dioxo-form [formula (1)] is not the only possible form of BR. In principle one may assume also the existence of the bislactim form



(5)

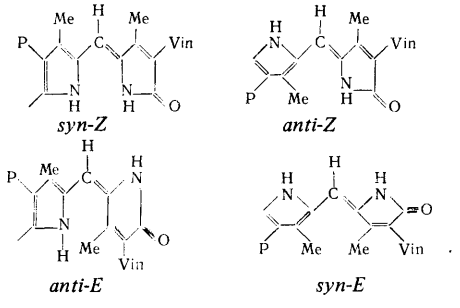
However, there are at present no reliable data concerning the content of a particular form both in solution and in the solid state. The bislactam form explains the majority of the chemical and physicochemical properties of BR but conflicts with the extremely low solubility of BR in water—at pH 7.4 and an ionic strength of 0.1 M the solubility of BR is only 0.05 mg litre^{-1}.[2] The contradiction disappears if one assumes the formation of a system of fairly strong intramolecular hydrogen bonds involving the propionic acid residues, the hydrogen atoms at the pyrrole nitrogen atoms, and the endocyclic carbonyl groups[3,4].

However, there are data showing that the hydrogen atoms at the pyrrole nitrogen atoms (and furthermore only in the two middle pyrrole rings) form hydrogen bonds only in non-polar solvents, while in the solid state of BR the four hydrogen atoms of the NH groups do not participate in the formation of intramolecular hydrogen bonds[5]. It has been suggested in addition that formula (6) corresponds to a planar conformation of the BR molecule[6], which has been confirmed by X-ray diffraction data[7]. Furthermore, the conformation of the BR molecule is chiral and its rigidity imparts to BR an optical activity[8]. The free energy barrier between the enantiomeric conformations of BR in chloroform, amounting to 17.9 ± 0.5 kcal mol^{-1}, has been determined by NMR.[9]



(6)

Together with structural and mirror isomerism, there is also a possibility of the *cis–trans* isomerism in the BR molecule, because the positions of the substituents relative to the two enamide C=C bonds can be different. The possible geometrical isomeric structures relative to only one of the C=C enamide bonds are illustrated below:



It has been shown by simple semiempirical conformational analysis[10] that the planar configuration holds only for the *syn-Z* isomer. Thus a structure of the type *syn-Z*, *syn-Z* should be ascribed to the planar conformation of the BR molecule. The isomerism associated with the rotation of the central pyrrole rings relative to the exocyclic C–C bond is usually neglected and the shorter designation *Z*, *Z* is used for the principal conformation of BR IXα.

2. Physicochemical Properties

Among the physicochemical properties of BR, its solubility in various media and the formation of complexes with other molecules are of greatest interest for photochemical investigations. Furthermore, the knowledge of these characteristics of BR is significant for the understanding of its behaviour in the organism at various metabolic stages.

The extremely low solubility of BR in water has already been mentioned. Since BR has two amide carbonyl groups and, what is still more significant, two carboxy-groups, the pH of the medium is of decisive importance for the solubility of BR in aqueous media. With increase of pH, the carboxy-groups ionise initially and then the enolic groups, which facilitates the solubilisation of BR in water. It is believed that 0.1 M NaOH, KOH, or Na_2CO_3 are optimal for the preparation of aqueous BR solutions. Potassium cyanide also promotes the dissolution of BR: 0.04 M BR dissolves in 0.1 M KCN.[6] When aqueous BR solutions are neutralised, colloidal solutions are formed. In acid solutions BR is almost insoluble. The majority of non-polar organic solvents do not dissolve BR. Polar solvents such as methanol, ethanol, and acetic acid are also included among solvents which are ineffective for BR. On the other hand, BR readily dissolves in acetone, benzene, isobutyl methyl ketone, formamide, dimethylformamide (DMF), dimethyl sulphoxide (DMSO), pyridine, and chloroform[6]. Chloroform has come to be particularly widely used by investigators.

A recently published communication[11] presents data contradicting the widespread concept of the lipophilicity of BR and its consequent neurotoxicity. The author[11] showed that the solubility of BR in triglycerides is low and that its solubility increases with increasing polarity of the medium.

The solubility of BR in aqueous solutions of proteins, particularly blood serum albumins, is of particular importance. It is noteworthy that proteins themselves are incapable of solubilising BR in aqueous solution. When it is necessary to dissolve BR in a neutral aqueous solution of a protein, BR is usually first dissolved in an alkine solution of the protein, which is then neutralised. It follows from this that proteins are incapable of disrupting the intramolecular hydrogen bonds in the BR molecule. On the other hand, proteins form fairly stable complexes with BR in which the above bonds had been ruptured beforehand[6].

Numerous studies have been made on the formation of complexes between BR and SA. It has been established that serum albumins possess centres for "strong" and "weak" binding of BR.[12,13] Between one and two BR molecules are strongly bound to SA and between 10 and 14 molecules are less strongly bound[13]. The less strongly bound BR molecules are comparatively readily displaced from the complexes by carboxylic acids[14–16] and also by certain other compounds.

3. Chemical Properties

In considering the structure of the BR molecule, a number of *a priori* conclusions can be reached concerning the most general possibilities for its chemical reactions. In the first place the BR molecule can be regarded as consisting of two conjugated fragments separated by a methylene bridge. Hence it is natural to suppose that the latter is a fairly active reaction centre, since, as a result of the possible dehydrogenation of BR at the bond adjoining this bridge, the conjugation embraces the entire tetrapyrrole system, which should lead to a gain in its energy. The presence of a large number of double bonds, including four exocyclic carbon–carbon double bonds, leads to the expectation of various addition reactions of BR involving the double bonds and also of oxidative cleavage reactions typical for olefins. The propionic acid residues are responsible for the weak acid properties of BR, which can be intensified in an alkaline medium as a result of the tautomeric transition of BR to the bislactim form with two enolic hydroxyls. The basic properties of BR are determined by the nitrogen atoms

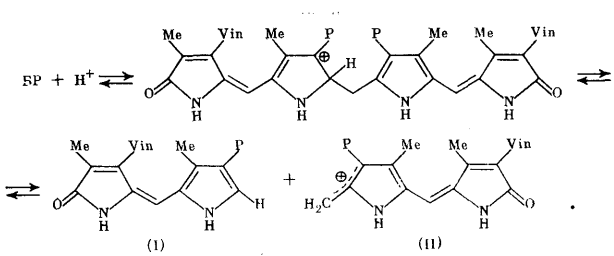
in the central pyrrole rings. In the extreme pyrrole rings the nitrogen atoms, which are of the amide type, are not basic.

Strong oxidants, such as potassium permanganate or chromic acid in acetic acid[17], decompose the tetrapyrrole system to monopyrrole compounds, from a mixture of which haematinic acid and methylvinylmaleimide were isolated. Weaker oxidants, such as iodine, a dilute solution of iron(III) chloride[18], cerium salts, or hydroquinone in an alkaline medium[19] oxidise only the central methylene bridge of BR with formation of a biliverdin molecule [see compound (V) below].

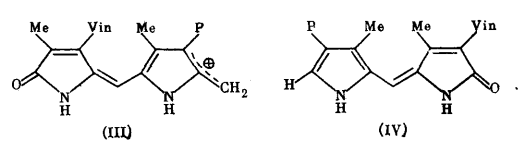
The reduction of BR is possible only with the aid of strong reductants. In the catalytic hydrogenation of BR in the presence of colloidal palladium or platinum in an alkaline medium[20], the vinyl group in the 18-position and then the vinyl group in the 3-position were reduced in succession (the formation of mesobilirubin), after which the $C_{(5)}$ and $C_{(15)}$ methylidene bridges were reduced under more severe conditions (the formation of urobilinogens). Sodium amalgam has also been used to reduce BR.[21,22].

The behaviour of BR in the presence of acids is interesting from all points of view. It has been observed[23] that, as a result of the acidification of a solution of BR IXα in DMSO with dry hydrogen chloride at room temperature, a mixture of BR isomers is formed after 1 min: IXα, IIIα, and XIIIα in proportions of 1:2:1. The isomerisation in the DMSO–acetic acid system takes place only on heating. In an alkaline medium (DMSO–NaOH–water) isomerisation was not observed.

On the basis of the products of the acid isomerisation of BR IXα, it is easy to postulate a process mechanism which apparently involves the protonation of the BR molecule at the $C_{(9)}$ or $C_{(11)}$ atoms with subsequent cleavage of the protonated BR molecule into two dipyrrolylmethene fragments. In particular, protonation at $C_{(9)}$ leads to the following transformations of BR IXα:



The protonation at $C_{(11)}$ similarly results in the formation of two fragments:



The recombination of (I) and (II) (the reverse reaction) leads to the reformation of the isomer BR IXα. On the other hand, the recombination of (I) and (III) and of (II) and (IV) leads to the BR isomers IIIα and XIIIα respectively.

A physiologically important reaction of BR is the formation of its water-soluble esters via the propionic acid residues, in particular the formation of esters with glucuronic acid. The mono- and di-glucuronides of BR have been obtained from BR with imidazole-activated carboxy-groups[24].

Numerous studies have been devoted to the formation of metal complexes of BR,[25–27] but these properties of BR are considerably outside the subject of the present review.

4. Optical Absorption Spectra

The optical characteristics, particularly the absorption spectra, of BR are important for the understanding of the mechanism of its photochemical reactions. According to data in a review[6], the absorption spectrum of a neutral aqueous solution of BR has a maximum at 440 nm, the transition to the alkaline region being accompanied by a significant hypsochromic shift (λ_{max} = 420 nm). When proteins are added to an alkaline solution of BR, the absorption maximum shifts towards longer wavelengths (450 nm), approaching the position of the maximum in a neutral protein-free solution. This factor suggests a definite similarity between the states of BR in a protein-free aqueous solution and in the presence of proteins. Since, as stated above, the formation of complexes between BR and proteins requires a preliminary dissociation of the intramolecular hydrogen bonds in the BR molecule, presumably the BR in complexes with proteins has no or virtually no bonds of this kind. On the other hand, the similarity of the spectra permits an analogous conclusion also concerning BR in a protein-free aqueous solution. The same state is apparently characteristic also of BR dissolved in chloroform (λ_{max} = 453 nm) and DMF (λ_{max} = 456 nm). The available data concerning the absorption of BR in the ultraviolet are contradictory owing to the different degrees of purity of the pigment investigated. One of the causes of this is the relatively low stability of BR in solutions. For example, the optical density at the BR absorption maximum in 0.1 M NaOH, NH_4OH, and Na_2CO_3 solutions decreases at a rate of 2–10% per hour[28]. It is therefore recommended that the ultraviolet and visible spectra of BR be measured in the course of several minutes after preparing the solution.

The infrared spectrum of BR in KBr contains the absorption band of the hydroxyl in the carboxy-group at 2500 cm^{-1} and of the carbonyl group at 1680 cm^{-1}. The bathochromic shift of the carbonyl band is due to the involvement of the carbonyl group in a hydrogen bond[29].

5. The Luminescent Properties

The luminescent properties of BR provide the best idea about the nature of the excited states of the molecule, which in its turn permits a deeper understanding of the photochemistry of BR and the associated problems of phototherapy. Neither the fluorescent nor the phosphorescence of BR solutions has been observed at room temperature. On the other hand, a greenish emission at ~525 nm has been noted in various polar solvents at 77 K.[30] The fluorescence quantum yield Q_F = 0.13 has been determined in ethanol at 77 K.[31]

The fluorescence of BR in the ether–isopentane–ethanol–DMF (6:5:3:0.5) system with excitation by the light of a high-frequency pulsing lamp at a wavelength <450 nm has been studied in detail[32]. The emission half-period was <5 ns, which is characteristic of fluorescence. The apparent fluorescence quantum yield (relative to fluorescein at room temperature) was ~0.05. The excitation spectrum, measured at the emission maximum at 520 nm, revealed a fairly sharp maximum at 472 ± 5 nm, which differed significantly from the broad band in the region of 470 nm in the absorption spectrum at room temperature. In their study of the influence of temperature in the range from 293

to 215 K on the absorption of BR in dichloroethane and DMF at 450 nm, the authors[32] observed spectral changes only for the solution in DMF. At the same time a fluorescence appeared in the DMF solution at 215 K, such fluorescence being absent from dichloroethane at the same temperature. On this basis, it was suggested[32] that a fluorescent form of BR is formed reversibly in certain polar solvents, particularly DMF, as the temperature is reduced.

Later it was observed that the fluorescence of a BR solution is possible also at room temperature when the solution contains SA with which BR forms a complex. Depending on the origin of the SA, both the emission maximum and the fluorescence quantum yield can vary[33]. Whereas in the case of human SA these parameters are respectively 527 nm and 0.00102, for bovine, rabbit, and monkey SA their respective values are 546 nm and 0.00085, 531 nm and 0.00084, and 528 nm and 0.00037. However, a higher fluorescence quantum yield of the complex of BR with bovine SA has been published[31] ($Q_F = 0.01$).

Thus a solution of BR, which does not fluoresce at room temperature, begins to fluoresce only when the temperature is reduced on formation of a complex of BR with a protein. In both cases light is emitted by a BR molecule, which has a limited number of degrees of freedom. Matheson et al.[31] therefore reached the fully logical conclusion that the radiationless loss of energy by BR in a liquid solution is determined by molecular vibrations or conformational changes, which completely suppress the emission. This conclusion has been to some extent confirmed by the observation of the fluorescence of BR in an aqueous solution in the micellar environment of cetyltrimethylammonium bromide molecules[34].

The absence of the fluorescence of BR under the usual conditions has been explained[35] by the rapid internal transition ($k \cong 10^{12}$ s^{-1}) of the excited singlet state S_1 formed initially into a state S_1' with a somewhat lower energy, which can undergo a radiationless transition either to the initial singlet state S_0 or to the first excited triplet state T_1, which will be considered later.

III. PHOTOCHEMICAL REACTIONS OF BILIRUBIN

1. Primary Photochemical Reactions

The photoexcited BR undergoes reactions giving rise to products which can in their turn undergo further reactions both under the influence of light and in the dark. In order to approach an understanding of the mechanisms of the photochemical reactions of BR, it is useful to consider separately the primary photochemical reactions and to attempt to isolate them from the overall chain of transformations of BR in the light. We shall introduce a more rigorous limitation by assuming that other molecules, including molecular oxygen, are not involved in the primary photochemical reactions of BR.

By irradiating with light from monochromatic and nonmonochromatic xenon sources a solution of BR in chloroform and also in phosphate buffer at pH 7.4, placed in a quartz cuvette, Davies and Keohan[36] observed by differential spectroscopy the presence of a photolabile product with an absorption maximum at 490 nm. The product could be detected only in the presence of an excess of BR, which played the role of a protecting material (internal filter). This experiment was subsequently reproduced by the same authors[37] using apparatus which permitted the isolation of the exciting emission with a maximum in the range 410–440 nm, having a low energy both below 360 nm and above 470 nm; in particular, this created conditions for a greater stability of the photoproduct with an absorption maximum at 490 nm. It was observed that the photoproduct concentration increases up to a BR conversion of 10–15% and then remains constant.

After a careful analysis of the differential spectra, the authors[37] concluded that, together with the photoproduct having $\lambda_{max} = 490$ nm, a second photoproduct, absorbing at 420 nm, is formed. The similarity of the differential spectra of seemingly different systems such as BR solutions in carbon tetrachloride and chloroform, on the one hand, and a solution of BR in phosphate buffer with added bovine SA, on the other, is noteworthy. In contrast to this, the differential spectrum of an irradiated BR solution in phosphate buffer with added human SA has its own characteristic features. Hence one may conclude that, in contrast to bovine SA, human SA modifies significantly human BR.

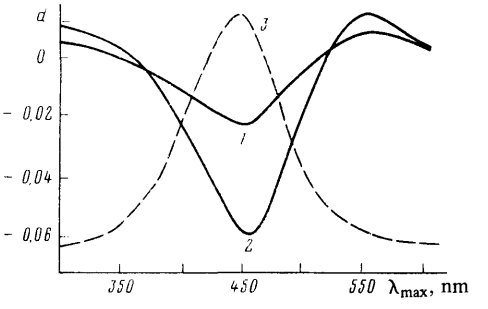


Figure 1. Differential spectra of a solution of BR in chloroform with 1% of ethanol on irradiation at a wavelength of ~440 nm: *1*) 60 s after irradiation; *2*) 360 s after irradiation; *3*) integral spectrum of BR (on an arbitrary scale).

On irradiating BR in chloroform with added 1% of ethanol at a wavelength of ~440 nm, Lightner et al.[38] observed a positive absorption in the region of 350 and 500 nm in the differential spectra (Fig.1) together with a negative maximum at ~460 nm (corresponding to a decrease of the amount of BR). Analogous spectra have also been obtained after irradiating BR solutions in chloroform with 1% of hexane at 420 and 430 nm and also in ammoniacal methanol at 400, 410, and 420 nm. It is significant that the absorption band at ~500 nm arises under both anaerobic and aerobic conditions. The absence of an effect by singlet oxygen in the latter case has been demonstrated by the weak influence on the spectrum by singlet oxygen fluorescence quenching agents—dimethylbutene, diethylamine, and triethylamine. The intensity of the band at ~500 nm gradually increases in the course of irradiation and reaches a constant value after 1–10 min (alkaline solvents accelerate the process). Under more prolonged irradiation, the intensity of this band begins to diminish. If a solution which has reached photoequilibrium is allowed to stand in the dark at 22 °C, a slow but quite appreciable thermal restoration of the initial spectrum takes place. This process is accelerated by the addition of traces of CF_3COOH (the reduction then takes place almost instantaneously) or I_2. An analogous effect also occurs on irradiation of a photoequilibrated solution by light at a

wavelength of ~510 nm with a band width of 10 nm. In the latter case a new photostationary state of the solution with a lower content of the photoproduct arises.

The authors[38] were able to isolate with the aid of thin-layer chromatography the photoproduct formed on irradiation of the BR solution in ammoniacal methanol. The product, which the authors called photobilirubin, consisted of a yellow substance with a broad absorption band at 440 nm but without an absorption peak in the region of 500 nm. Photobilirubin was always contaminated with different amounts of BR, which the authors believe is formed via a reverse thermal transformation of photobilirubin into BR. According to Lightner et al.[38], the peak at 350 and 500 nm in the differential spectra are not absorption maxima of the photoproduct and merely characterise the difference between the absorption of BR and that of the photoproduct in the region of the above wavelengths. The logical hypothesis was put forward that photobilirubin is none other than a mixture of the photoisomerisation products of BR involving the exocyclic C=C enamide bonds, among which the virtually identical *Z*, *E*-, and *E*, *Z*-isomers predominate and there is a small amount of the *E*, *E*-isomer (see the formulae above).

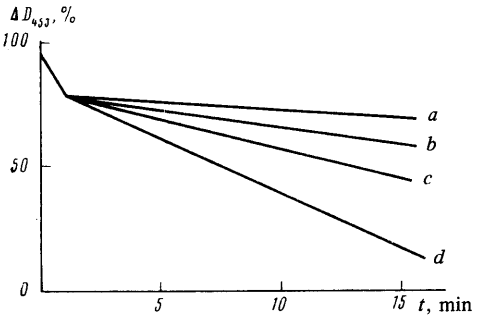


Figure 2. Decrease of the concentration of BR on irradiation of its solution in chloroform (at a concentration of 1.5×10^{-2} M) from a light source at a wavelength >390 nm (for explanation, see text).

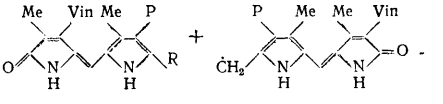
Fig. 2 presents curves characterising the decrease of the BR concentration (decrease of absorption at 453 nm) observed in chloroform solution by light at a wavelength >390 nm.[38] Solutions saturated with argon (*a*), saturated with O_2 in the presence of 0.15 M 1,4-diazabicyclo[2,2,2]octane (*b*), saturated with O_2 in the presence of 10^{-3} M dimethylbutene (*c*), and simply saturated with O_2 (*d*). Evidently all the curves have a common initial section, to which corresponds an irradiation time of ~1 min. The authors[38] suggest that this section characterises the formation of photobilirubin, while the subsequent sections of the individual curves characterise the conversion of BR, the rate of which depends on the mixture composition.

Products similar to the photobilirubin found by Lightner et al.[38] have been discovered also by other workers. In particular, Zenone[39] et al., who irradiated BR in chloroform with visible light, isolated in 7% yield a yellow product with a higher polarity than BR IXα. After irradiating with intense visible light a solution of BR in chloroform, Stoll et al.[40] isolated three BR isomers having the same absorption maximum (442 nm) as BR IXα (the *Z*, *Z*-isomer). Two of these isomers decomposed in the dark with formation of brown products, while the third was reconverted into the initial BR. On irradiation, all three isomers were converted into the *Z*, *Z*-isomer BR IXα.

The studies discussed above permit a conclusion very important for the understanding of the photochemistry of BR: the primary products arising on irradiation of BR with visible light are its geometrical isomers. Possibly one of the causes of the absence of the BR fluorescence and phosphorescence under the usual conditions is the consumption of the excess energy of the excited singlet and triplet states on overcoming the energy barrier separating the *Z*, *Z*-isomer of BR from the other geometrical isomers. Being less stable than the *Z*, *Z*-isomer, these isomers are either reconverted into the initial BR or are irreversibly converted into non-BR products, depending on the conditions. A particularly important property of the BR "photoisomers" is their polarity, which is apparently determined by the complete or partial rupture of the intramolecular hydrogen bonds characteristic of the *Z*, *Z*-isomer of BR. The polarity is responsible for the water solubility of these isomers, which may prove to be one of the decisive factors in the phototherapy of the jaundice of the new-born.

2. Photochemical Formation of Structural Isomers of Bilirubin

As already stated, in the presence of an acid BR IXα isomerises to its structural isomers IIIα and XIIIα. The first stage of the process is the protonation of the BR molecule at the $C_{(9)}$ or $C_{(11)}$ atoms. On the basis of general chemical considerations, one may assume that an analogous reaction can proceed also via a radical mechanism. If a free radical $\dot{R}$ arises for one reason or another in a solution containing BR IXα, one of the possible reactions will be its addition to the BR molecule at $C_{(9)}$ or $C_{(11)}$. Each of the newly formed tetrapyrrole free radicals can decompose into two dipyrrole fragments, for example [after addition to $C_{(9)}$]:



The radical fragment can in its turn add to an unreacted molecule of BR IXα at $C_{(9)}$ or $C_{(11)}$ (chain propagation), after which the dipyrrole fragment, incorporated in the initial molecule, can be split off in the form of a free radical from the hexapyrrole free radical formed, etc. As a result, a further two isomers (IIIα and XIIIα) will be produced instead of the isomer BR IXα present initially in this system. The primary radical $\dot{R}$ can be formed from any one component of the system, including a BR molecule. One of the factors stimulating the generation of free radicals is irradiation by light.

Photoisomerisation of the type IXα ⇌ IIIα ⇌ XIIIα has indeed been frequently observed experimentally. In particular, McDonagh[41] observed this isomerisation after irradiating with visible light solutions of BR IXα (2.5 mg %) in aqueous buffers at pH 8.0 and 8.5 in the absence of O_2. The reaction proved to be less typical for a solution of BR in chloroform and did not occur at all in 0.1 *N* aqueous NaOH solution in DMSO.

The photoisomerisation of BR IXα to its structural isomers is probably one of the causes of the usual contamination of commercial BR preparations by isomers IIIα and XIIIα.

3. Interaction of Bilirubin with Molecular Oxygen on Photoirradiation

Since the photochemical reactions of BR under physiological conditions always take place in the presence of molecular oxygen, studies of the photochemistry of BR in oxygen-containing solutions and, specifically the interaction of BR with O_2 under photoirradiation conditions, are of greatest interest from the practical point of view.

McDonagh[42] was the first to show that BR sensitises the formation of singlet oxygen. He established that, when BR is irradiated with visible light in chloroform, its photochemical oxidation is greatly accelerated by Methylene Blue, octaethylporphin, and other agents photosensitising the formation of $^1O_2$, while $^1O_2$ quenching agents—1,4-diazabicyclo[2,2,2]octane and *trans*-β-carotene—inhibit photochemical oxidation. McDonagh[42] suggested that the photochemical oxidation of BR by molecular oxygen proceeds via a stage involving energy transfer from the excited triplet BR to $^3O_2$. Another piece of evidence for the participation of singlet oxygen in the reaction (predominantly in the $^1\Delta g$ state) is the similarity of the rate constant for the reaction of BR with photophysically generated $^1O_2$ in water (3.5×10^8 litre mol^{-1} s^{-1}) to the rate constants for the photochemical oxidation of BR in chloroform, a mixture of chloroform and methanol, and other solvents. The rate constant for the physical quenching of the fluorescence of bilirubin by $^1O_2$ has been determined as 9×10^8 litre mol^{-1} s^{-1}.[37]

The existence of the triplet state of BR has been demonstrated experimentally[43]. A solution of BR at a concentration of 4×10^{-5} M in benzene with added 0.1 M biphenyl was subjected to pulse radiolysis with a dose of ~1000 rad, after which the absorption spectrum of the excited BR was measured. It was observed that, over the entire range of the experimental spectrum (490–700 nm), after a 20 ns excitation pulse the absorption intensity uniformly increases to a maximum after 5 μs, and then decreases with a half-life of 9 μs. The rate of increase of absorption is the same as the rate of decomposition of the triplet state of biphenyl measured at 360 nm.

An analogous but weaker absorption arises also in the pulse radiolysis of BR in the absence of biphenyl. After adding 10^{-5} M BR to a solution of anthracene in benzene (at a concentration of 3×10^{-5} M), the author[43] noted an appreciable increase of the rate of decomposition of the triplet state of anthracene, whence it follows that the triplet level of BR is lower than that of anthracene. It has also been established that the triplet level of BR is close to that of perylene and has an energy of ~150 kJ mol^{-1}. The yield of the singlet–triplet transition $\phi_T \leq 0.1$ has been determined by laser flash photolysis using anthracene as a standard. The proposed mechanism of the photochemical oxidation of BR includes the following stages:

$$\mathrm{BR} \rightarrow {}^1\mathrm{BR}^*,$$

$${}^1\mathrm{BR}^* \rightarrow {}^3\mathrm{BR}^*,$$

$${}^3\mathrm{BR} + \mathrm{O}_2({}^3\Sigma_g^-) \rightarrow \mathrm{BR} + \mathrm{O}_2({}^1\Delta_g),$$

$$\mathrm{O}_2({}^1\Delta_g) + \mathrm{BR} \rightarrow \text{BR oxidation products}.$$

Since the energy of the $O_2(^1\Delta_g)$ molecule is 94 kJ mol^{-1} and the energy of ^{3}BR* is ~150 kJ mol^{-1}, the reverse energy transfer from $O_2(^1\Delta_g)$ to BR in the ground state with excitation of BR in the triplet state is impossible. The quenching of singlet oxygen in its interaction with BR is therefore accompanied by chemical transformations of BR. On the other hand, agents quenching singlet oxygen, having an energy between 150 and 94 kJ mol^{-1}, can inhibit the decomposition of BR, as suggested by Land[43]; the inhibition takes place via energy transfer from the triplet state of BR. Quenching agents with energies less than 94 kJ mol^{-1} can inhibit the decomposition of BR both as a result of energy transfer from its triplet state and as a result of the quenching of the singlet oxygen (depending on the relative concentrations of O_2 and the quenching agent). As regards sensitisers, those whose energy exceeds 150 kJ mol^{-1} can facilitate the formation of ^{3}BR* mainly before the sensitisation of the formation of $O_2(^1\Delta_g)$. On the other hand, sensitisers with energies between 150 and 94 kJ mol^{-1} can only excite the formation of $O_2(^1\Delta_g)$.

Matheson et al.[35] investigated the influence of various factors on the quantum yields (ϕ) of the photochemical oxidation of BR using its dilute solutions—$(0.5–5) \times 10^{-3}$ M. The latter factor prevented the formation of biliverdin, which is favoured by high BR concentrations. It was established that ϕ increases with increasing O_2 concentration in chloroform, methanol, Freon-113, and water. In aqueous solution ϕ increases with increase of pH from 7 to 11. The following mechanism of the photochemical transformations of a general singlet oxygen acceptor (A) has been proposed:

$$\mathrm{A} \rightarrow {}^1\mathrm{A}\ ,$$

$${}^1\mathrm{A} \xrightarrow{k_1} \mathrm{A}\ ,$$

$${}^1\mathrm{A} \xrightarrow{k_2} {}^3\mathrm{A}\ ,$$

$${}^1\mathrm{A} + {}^3\mathrm{O}_2 \xrightarrow{k_3} {}^3\mathrm{A} + {}^3\mathrm{O}_2\ ,$$

$${}^1\mathrm{A} + {}^3\mathrm{O}_2 \xrightarrow{k_4} {}^3\mathrm{A} + {}^1\mathrm{O}_2\ ,$$

$${}^3\mathrm{A} + {}^3\mathrm{O}_2 \xrightarrow{k_5} \mathrm{A} + {}^1\mathrm{O}_2\ ,$$

$${}^3\mathrm{A} \xrightarrow{k_6} \mathrm{A}\ .$$

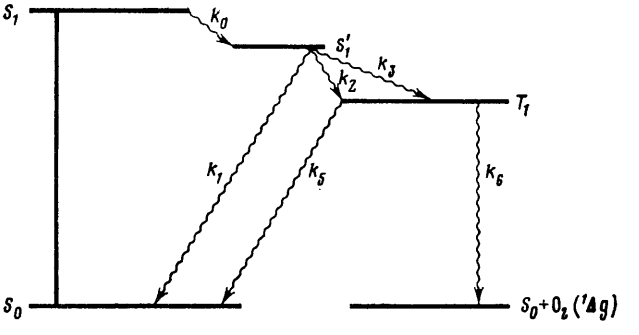


Figure 3. The energy levels of BR on photoexcitation in the presence of O_2; transitions with the rate constants k_3 and k_4 take place in the presence of $^3O_2$.

In the case of BR the rate constant k_4 can be neglected, since the energy of the singlet–triplet cleavage for BR (the difference between the energy of ^{1}BR*, which is 240 kJ mol^{-1},[31] and the energy of ^{3}BR*, which is ~150 kJ mol^{-1} [43]) is ~90 kJ mol^{-1}, which is less than the energy of $O_2(^1\Delta_g)$ (94 kJ mol^{-1}). The constant k_3 is largely determined by diffusion and should therefore be much less than

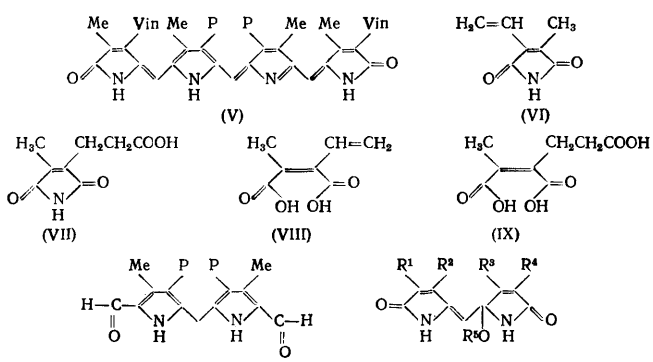
k_1. However, this is true only in chloroform solution, while in other solvents $k_3 > k_1$. On this basis, the authors[35] concluded that the state relaxing with the rate constant k_1 is not the excited singlet state formed initially. A scheme of the interlevel transitions in the photoexcitation of BR and in the relaxation of the excited BR state in the presence of O_2, illustrated in Fig.3, has been proposed. According to this scheme, the state S_1 formed initially is very rapidly converted into the non-fluorescent state S_1'.

The quantum yields of the excitation of BR to the triplet state in various media have been measured in a recently published study[44] (using laser flash photolysis at a wavelength of 347 nm). In particular, in an aqueous solution at pH 9–11 and in ammoniacal methanol the quantum yield was less than 0.005. In a non-polar medium (benzene) $\phi \cong 0.001$. The addition of triethylamine to benzene lowered ϕ to less than 0.005.

4. Photochemical Oxidation of Bilirubin

Many workers have frequently noted that the photodegradation of BR in the presence of atmospheric oxygen proceeds much more rapidly than in its absence. As already stated in a previous section, the first stage of the photochemical oxidation of BR is the photoexcitation of its molecule to the triplet state (arising as a result of the intercombination transition from the excited singlet state of BR). The half-life of the state (9 μs) is entirely sufficient for the transfer of the triplet energy to the molecular oxygen. The singlet oxygen formed under these conditions can react in its turn with a BR molecule in the ground state, oxidising it. Thus BR is, on the one hand, a photosensitiser of the formation of molecular oxygen and, on the other hand, it is an acceptor of singlet oxygen. The photochemical oxidation of BR by molecular oxygen has therefore come to be regarded as self-sensitised.

The composition of the products of the photochemical oxidation of BR depends greatly on the medium in which the process takes place. In protic solvents (usually in ammoniacal methanol, ethanol, and water) different workers have detected biliverdin (biliverdin IX) (V), methylvinylmaleimide (VI), haematinimide (VII), methylvinylmaleic acid(VIII), haematinic acid (IX), dipyrroledialdehyde (X), and the adducts of propentdyopents (XI), whose formulae are given below:

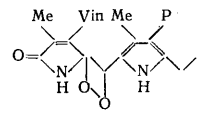


(V) (VI) (VII) (VIII) (IX) (X) (XI)

(XI): (a) R^1, R^3=Me; R^2=Vin; R^4=P; (b) R^2, R^3=Me; R^1=Vin; R^4=P: (c) R^2, R^3=Me; R^4=Vin; R^1=P; (d) R^2; R^4=Me; R^3=Vin; R^1=P; R^5=H, Me or Et.

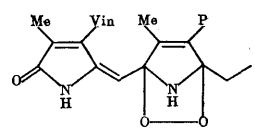
The green pigment biliverdin is the product of the oxidation of the methylene bridge in the BR molecule, which can be achieved fairly readily and occurs even in the dark, albeit at a low rate[45]. The formation of biliverdin from BR also in the absence of oxygen is known[46]. The ease of the conversion of BR into biliverdin served as a basis for the hypothesis that biliverdin is an intermediate in the more far-reaching photodegradation of BR.[47,48] However, there is serious evidence against this claim. After irradiating the dimethyl ester of biliverdin in chloroform (biliverdin itself is sparingly soluble in chloroform), McDonagh[49] established that the biliverdin ester does not sensitise the excitation of oxygen. Comparative experiments with irradiation by visible light showed that the photodegradation of BR is significantly faster than that of the dimethyl ester of biliverdin[49,50]. On the other hand, it has been established that the dimethyl ester of biliverdin is in the first place, like BR, a quenching agent for singlet oxygen and, in the second place, a much poorer $^1O_2$ acceptor than BR.[49] Being a $^1O_2$ quenching agent while at the same time undergoing very slow oxidation, biliverdin therefore inhibits the photochemical oxidation of BR, while the main BR oxidation pathway does not involve the intermediate formation of biliverdin.

Methylvinylmaleimide is formed[47,51] as a result of the attack by singlet oxygen on the exocyclic C = C bond in the 4- or 15-position in the BR molecule with the intermediate formation of a four-membered peroxide ring:



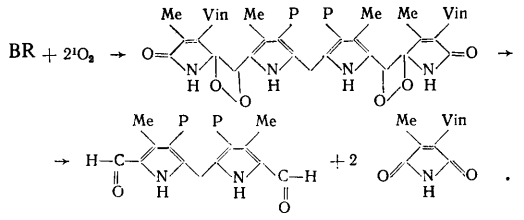
While the detection of methylvinylmaleimide *in vitro* in alcoholic solutions suggested that it can be formed also in the organism, this hypothesis became more definite after its detection also in a number of BR photochemical decomposition products in an aqueous buffer[52]. Under the influence of light, methylvinylmaleimide undergoes further degradation and the process takes place even in the absence of oxygen, albeit more slowly. The methylvinylmaleimide degradation products have not been determined, but it has been suggested that they can be oxidised to methylcarboxymaleimide, the decarboxylation of which can lead in its turn to the formation of methylmaleimide[53]. The latter is a modifying agent for proteins and can interfere with their normal functioning in the organism.

The formation of the imide of haematinic acid is apparently preceded by the interaction of O_2 (dienophile) with the conjugated diene system of one of the central pyrrole rings in the BR molecule, which results in the formation of a peroxide adduct having the following structure[54]:

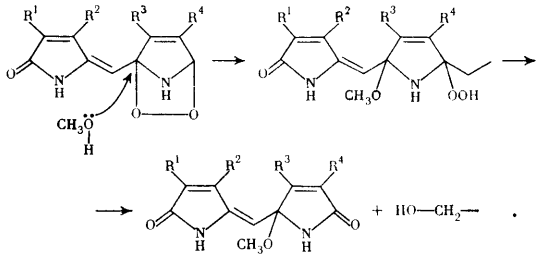


The appearance of methylvinylmaleic and haematinic acids in solutions is a natural consequence of the hydrolysis of the corresponding imides either in the photochemical reaction or in the treatment of the reaction mixture. The dipyrroledialdehyde detected in the reaction mixture[50,54] can be formed by a reaction of the same type as that leading to the formation of methylvinylmaleimide: the interaction of molecular oxygen with the double bonds in the 4- and 15-positions of BR. Theoretically the formation of a single dipyrroledialdehyde molecule is accompanied by the formation of two molecules of

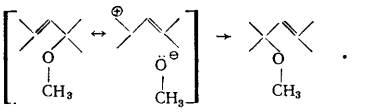
methylvinylmaleimide[55]:



A considerable proportion of the BR photochemical oxidation products in protic solvents consist of adducts of propentdyopents with the corresponding solvent. The mechanism proposed[56] for the formation of adducts of propentdyopents in an ammoniacal methanol solution includes the preliminary formation of an endoperoxide—the product of the diene condensation of O_2 with the diene system of the second or third pyrrole ring—and subsequent methanolysis of the endoperoxide and the decomposition of the intermediate hydroperoxide:



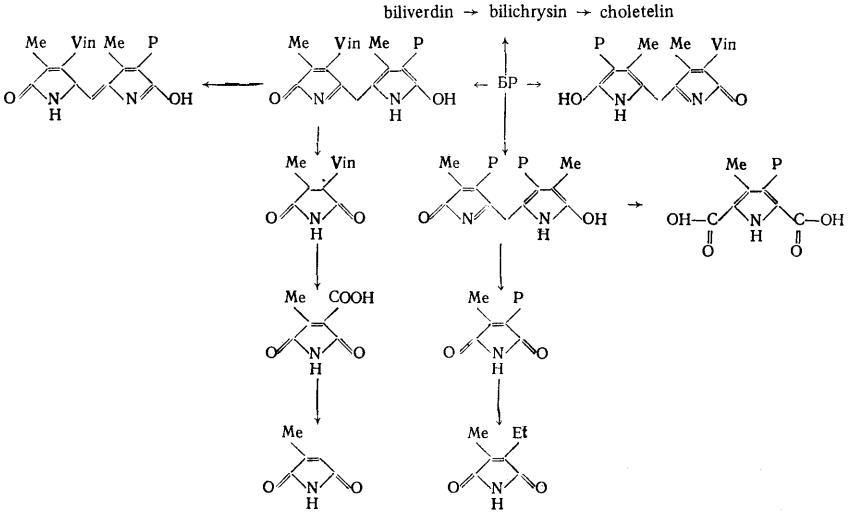
Bearing in mind that the irradiation of BR IXα with light results in the formation of its equilibrium mixture with the isomers IIIα and XIIIα, it follows that, in conformity with the above scheme, each BR isomer should form on irradiation in methanol the corresponding methanol adducts of propentdyopents: IXα–propentdyopents (a and b), IIIα–propentdyopent (b), and XIIIα–propentdyopent (a). However, the formation of propentdyopent (a) was not confirmed in a later study[51]. Together with product (b), its isomers (c) and (d), which are not formed directly from BR IXα and its two isomers via the scheme proposed by Bonnett[56], were detected in the reaction mixture. In model experiments the propentdyopents (b) and (c) were obtained from the pure isomer BR IIIα, while propentdyopent (d) was obtained from BR XIIIα. Thus the formation of a mixture of propentdyopents (b), (c), and (d) from the irradiated BR IXα can be explained, on the one hand, by the photoisomerisation of BR IXα to IIIα to XIIIα and, on the other hand, by the isomerisation of the methanol adducts of the propentdyopents already formed, consisting in the transfer of the methoxy-group via one carbon atom to a neighbouring pyrrole ring with the simultaneous migration of the double bond (the allyl rearrangement):



The isomerisation of the propentdyopent adducts isolated has been confirmed experimentally[51]. On the other hand, the absence of propentdyopent (a) from the reaction mixture is apparently due to the marked shift of the equilibrium (a) ⇌ (d) to the right.

Numerous studies have been made on the photochemical oxidation of BR using its solutions in chloroform[28,42,53,57]. The appearance, together with biliverdin, of vicinal diols, formally representing the products of the hydroxylation of biliverdin at the non-vinyl exocyclic C=C bonds, has been noted in this solvent[57,58]. It has been suggested that the above compounds are produced as a result of the interaction of the excited BR with triplet oxygen. A smaller yield of the di- and mono-pyrrole oxidation products, compared with that observed in reactions in protic solvents was also noted[59,60]. Fundamental differences between the nature of the photochemical oxidation of BR in solutions in chloroform and carbon tetrachloride were not observed despite the difference between the polarities of these solvents. The differential spectra characterising the changes in these solutions on irradiation with light having a band maximum at 410–440 nm were in broad outline the same[37].

In a study of the photochemical oxidation of BR in chloroform under the influence of scattered sunlight over a long period (500 days), Gray et al.[53] isolated various products at different stages of the process, which enabled them to set up a scheme of the probable transformations of BR (see below).



The authors[53] established with the aid of paper chromatography that BR disappears completely from the reaction mixture after 12–25 days (depending on the intensity of illumination). Biliverdin, whose concentration in the mixture never exceeded 13% of the initial BR concentration, disappeared after 40–50 days. On the hundredth day the solution became almost colourless. The observed stability of BR in chloroform under a nitrogen atmosphere led to the conclusion that most of the BR decomposes via oxidation with molecular oxygen.

Gray et al.[53] also compared the BR photodegradation pathways in various solvents. In particular, they noted that in a methanol–chloroform mixture, weakly alkaline methanol and aqueous solutions, BR decomposes with rapid breakdown of the tetrapyrrole structure, as in chloroform solutions. On the other hand, in acid media the decomposition of the tetrapyrrole structure proceeds with much difficulty: propentdyopents, imides, and dipyrrolylmethenes were detected only in weakly acid solutions. Furthermore, the overall rate of decomposition of the BR in acid solutions is lower than in alkaline solutions.

A number of studies have been devoted to the photochemical oxidation of BR in the presence of serum albumin[36,48,61]. On the one hand, a complex of BR with SA is formed in a living organism and is of special interest. On the other hand, this complex is soluble in water, which makes it possible to investigate the behaviour of BR on photoirradiation in neutral aqueous solutions. By varying the pH of the medium in the range 7.4–13.0, Ostrow and Branham[48] established that in a neutral medium SA accelerates the photochemical decomposition of BR, while in an alkaline medium it retards it. It has also been noted[61] that BR becomes more polar in the course of irradiation and that its bond with the protein is consequently weakened. The photochemical oxidation of SA was not observed at the given stage. However, by irradiating the complexes of BR with human and bovine SA in aqueous solution at pH 7.4, Rubatelli and Jori[62] observed changes, occurring as a result of the photodynamic effect, apparently in the two histidine residues in both albumins. The authors suggest that these changes do not alter the tertiary structure of the proteins but decrease their binding capacity in relation to BR. It was also noted that the tertiary structure of bovine SA is more labile than that of human SA.

As already mentioned, the primary photoproducts of the complexes of BR with human and bovine SA are different: bovine SA does not have a specific effect on the nature of the photochemical transformation of BR, while in the complex with human SA BR undergoes an extremely specific photochemical transformation. Complexes of BR dissolved in the serum of rats suffering from congenital and stimulated jaundice also did not exhibit specific features.

IV. PHOTOTHERAPY OF NEONATAL JAUNDICE AND ITS PROBLEMS

The use of light for the treatment of neonatal jaundice began to be practised after it was observed[63] in 1958 that BR in the serum of a jaundiced infant decomposes in the light. Clinical experiments demonstrating the absence of a neurotoxic effect of the BR photochemical degradation products served as a basis for the practical feasibility of the phototherapy of neonatal jaundice[64,65]. It has also been established[66] that a decrease of the level of BR in the organism during phototherapy is caused not only by the photochemical degradation of BR but also by a change in the nature of the elimination of the undecomposed BR: whereas under the usual conditions BR is eliminated from the organism mainly in a bound state, in phototherapy there is a sharp increase in the amount of unbound BR in the bile, while the elimination of BR in a bound form diminishes. As already mentioned previously, this may be a consequence of the *cis–trans* isomerisation of BR with formation of its water-soluble geometrical isomers[38].

As was to be expected, light in the wavelength range 450–600 nm, corresponding to the BR absorption maximum, exhibits the greatest effect in phototherapy[67]. However, a small effect has also been observed on irradiation with light at a wavelength of ~550 nm and also on ultraviolet irradiation[67].

Despite some contradiction between the results reported by different workers, in most instances successful phototherapy has been achieved by irradiating patients from luminescent sources of blue and white light with a power of 20–40 W, between 3 and 12 of which are placed at a distance of 50–100 cm. More details recommendations concerning the phototherapy of neonatal jaundice are given in a review[68].

The site of the photochemical degradation of the BR molecule on irradiation of a jaundiced infant has not been unambiguously established. Some light has been thrown on this problem by a study[59] of the effect of the shading of individual small (up to ~4 cm^2) sections of the skin in phototherapy. These sections proved to be more pigmented in all cases than the exposed sections of the skin. Hence it follows that BR is firmly retained in the intervascular space and that the "bleaching" effect in phototherapy is due mainly to the decomposition of BR in the surface layers of the skin and not in the vessels. In view of the diffuseness of the boundary line between the shaded and exposed sections of the skin, it was suggested that the effect of light extends along the skin over a distance of ~2 mm. Vogl[69] also claims that BR leaves the vessels more readily than it is able to return to them. It has also been reported[70] that the yellow colour of the skin disappears in the course of phototherapy, while the BR level remains unchanged in the organism. Consequently one may assume that the decomposition of BR in the skin under irradiation is faster than the supply of BR from the blood vessels to the skin.

A group of Indian investigators[71] observed in *in vitro* experiments that the epidermis and skin of man absorbed free and serum-bound BR from an aqueous buffer. After this, the bound BR was readily removed when the skin was transferred to a medium free from BR, while the free BR remained in the skin, imparting a yellow colour to the latter. If the skin was irradiated subsequently with the light from a mercury lamp, then the BR decomposition products accumulated in the medium. Using BR labelled with ^3H, Kapoor et al.[71] established an extremely significant fact: the products for the oxidation of BR absorbed by the skin differ from the products of the oxidation of BR present in the buffer solution. It was suggested that, after emerging from the vessels, BR is bound by the skin lipids and collagen, in which the BR concentration can exceed its concentration in plasma.

The problem of the possible complications in phototherapy has been discussed from the very beginning of its application. In the study of children subjected to phototherapy no arrest was observed in their growth or anomalies in their psychological or physical development[72,73]. Nevertheless, in view of the importance of the problem, special consideration must be given to a number of clinically observed and potential complications.

Complications in phototherapy in which a greyish-brown colouring of the skin, serum, and urine was observed have been reported. This phenomenon may be complicated by anaemia. Cases are known where premature new-born babies, suffering from hyperpigmentation of the skin and subjected to irradiation, showed an arrest of growth[74,75]. An instance of acute haemolysis, accompanying the hyperpigmentation of the skin, on irradiation of a premature infant with daylight lamps has been described[76].

The possibility of the haemolysis of erythrocytes on irradiation in the presence of BR cannot be ruled out. As early as 1932 it was shown[77] that erythrocytes suspended in BR solutions haemolyse on irradiation with sunlight. Subsequently[78] it was suggested that BR is bound to erythrocytes and on irradiation can exert a photodynamic effect on the latter. However, the haemolytic effect of BR can be partly inhibited by proteins[79].

For high doses of irradiation by a luminescent lamp of a suspension of erythrocytes containing BR and at concentrations characteristic of the neonatal period, it was observed[80] that haemolysis is preceded by damage to erythrocyte membranes resulting in the loss of potassium by erythrocytes and a decrease of the activity of ATPase. When BR was introduced into erythrocytes at pH 8 and 10 °C, it sensitised the peroxide oxidation of unsaturated lipids on irradiation with blue light, which was inferred from the positive colour reaction with 2-thiobarbituric acid[81]. The peroxide oxidation of lipids was accompanied by the disappearance of a large proportion of the structural proteins of the membranes and by the appearance of polypeptide photoproducts with a molecular weight in excess of 25000. It was suggested that the photodynamic effect of BR *in vivo* may involve not only erythrocytes but also other cells coming into contact with BR. The photodynamic damage to SA [62] was mentioned above.

Another serious complication in phototherapy may be damage to DNA, which, however, is still one of the problems which have been little investigated. A change in the physicochemical properties of single-stranded DNA *in vitro* has been observed[83] after its irradiation in the presence of BR with light at a wavelength of 450 nm. Bearing in mind the possible relation between the ability to modify cell DNA and the potential mutagenic and/or carcinogenic effect, Speck and Rosenkranz[82] concluded that the possibility of dangerous aftereffects must be completely eliminated in phototherapy.

The appearance of a slight rash, an irritation of the skin, an increase of temperature[77], and an increased loss of water through the skin[83] have been observed in some children on phototherapy. Cases of the tanning of the skin in negro children have been described[84].

In 1974 the National Committee on Phototherapy in the USA suggested in its preliminary report[84] that the phototherapy of neonatal jaundice be regarded as an extremely effective albeit still an experimental method. At the present time most of the large maternity establishments in many countries have already been provided with phototherapeutic apparatus and phototherapy is one of the main methods of preventing the severe and irreversible consequences of neonatal jaundice. Some investigators[85] actually recommend that phototherapy be used as a prophylactic procedure for all new-born infants. However, the majority of investigators nevertheless recommend that phototherapy should be resorted to selectively—only in those cases where the necessity for phototherapy has been firmly established on the basis of the analysis of blood for BR.

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