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Complex-Formation and Redox Reactions of Bilirubin and Biliverdin with Zinc(II), Cadmium(II) and Copper(II) Ions

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Abstract

Transition metal complexes of bilirubin and biliverdin were studied spectrophotometrically, in DMSO and in a boric acid–NaOH buffer mixture at pH 10.5. In the zinc(II) and cadmium(II)–bilirubin systems, 2:1 complexes are formed. Both in aqueous and in DMSO medium, the copper(II) ion oxidizes bilirubin to biliverdin. With all three metal ions, biliverdin forms 1:1 complexes, the stabilities of which are higher than those of the corresponding bilirubin complexes. Accordingly, these metal ions accelerate the oxidative transformations of bilirubin.

Introduction

Very wide-ranging studies have long been made on the possible biochemical transformations of bilirubin, (for structure see Fig. 3 and Structure I) which is formed during the decomposition of haemoglobin. Particular attention has been paid to its photochemical and redox reactions. Results are also available on the interactions between metal ions and bilirubin, but the relevant publications comprise only a very small proportion of those dealing with the biochemistry of bilirubin. At the same time, D-penicillamine is well known to display a strong tendency to bind metal ions [1], and its activity in the treatment of neonatal jaundice [2] suggested the possibility of a direct or indirect biochemical role to the interaction between bilirubin and metal ions.

The possibility of complex formation between transition metal ions and bilirubin was suggested long ago, but the earlier data are fairly contradictory [3–5]. It was pointed out by Van Norman and Szentirmay [6] that the cause of these contradictions was presumably the lack of acid–base considerations. They studied the Zn(II)–bilirubin interaction in non-aqueous medium in the presence of proton-binding substances, and concluded that a 2:1 complex is formed [7]. Van Norman and Yatsko [8] later found that there are 3 possible modes for the metal ion–bilirubin interaction:

(a) One group of metal ions (Zn(II), Cd(II), Ni(II)) form 2:1 complexes by coordination of the pyrrole N atoms, the bilirubin absorption bands undergoing a bathochromic shift.

(b) Another group of metal ions (Al(III), Pb(II), Ag(I), Co(II)) are coordinated via the propionic acid side-chain, which results in a hypsochromic shift.

(c) The interaction between Cu(II) and bilirubin is 'irreversible', presumably because of a redox reaction.

Although the above results are based on a large amount of careful experimental work, they raise a number of unsolved questions. For instance, the 'non-systematic' classification of the transition metal ions into the various groups is especially surprising. It therefore appears necessary to carry out wideranging studies on the bilirubin complexes of metal ions. As a consequence of the possible redox reactions, it is advisable that these studies should be extended to the interactions with the oxidized forms of bilirubin, and above all biliverdin. For a reliable evaluation of the results, it is also necessary to make measurements in both inert and oxidative atmospheres and, as far as possible, in both aqueous and non-aqueous media.

In the present paper we report results on the copper(II), zinc(II) and cadmium(II) complexes in accordance with the above aims.

Experimental

The bilirubin (BR) used was a Reanal product of the analytical purity grade (ϵ_{455} = 60,000 ± 1,000 mol^{-1} cm^{-1} dm^3 in chloroform). The biliverdin, 2HCl (BV) was a Sigma product of 80% purity.

The studies in aqueous medium were made in a boric acid–NaOH buffer mixture at pH 10.5. For the preparation of stock solutions, the necessary amount of bilirubin was dissolved in a diluted NaOH solution in an inert atmosphere, and the pH was

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then adjusted to 10.5 with buffer. In general we worked with 3×10^{-4} mol dm^{-3} solutions, which were stored in the dark. Biliverdin dissolves directly in the pH 10.5 buffer, and the stock solutions were therefore prepared by direct weighing and dissolution.

Studies in DMSO were similarly made with 3×10^{-4} bilirubin and biliverdin solutions. Dissolution was performed in N_2 atmosphere. 3×10^{-3} mol dm^{-3} solutions of the metal ions were used in chloride form. Solutions of tetramethylammonium hydroxide (TMA), Na-phenolate and Na-acetate in DMSO were used as 'proton-binding' substances.

Spectrophotometric measurements were made on a Beckman Acta MIV spectrophotometer. Spectra were recorded in 1 mm cells, in the interval 350–800 nm. Investigations in an inert atmosphere were performed in a closed system coupled with a flow-cell. A laboratory-built reciprocating pump was used to circulate the reaction mixture.

Results and Discussion

1. Complexes of Zinc(II) and Cadmium(II) Ions in DMSO Medium

It is stated in the literature [7] that zinc(II) forms a 2:1 complex with bilirubin, coordination involving the pyrrole N atoms. This compound was reported to form only in the presence of proton-binding substances (*e.g.* tetramethylguanidine), in dimethylformamide.

Our measurements in DMSO as solvent led to results conforming with the earlier observations, *i.e.* we did not observe a direct interaction between bilirubin and zinc(II) or cadmium(II) ions. The addition of Na-acetate caused a spectral shift in the red direction, which is an indication of complex formation. Under such conditions, however, the transformation is not complete, *i.e.* the acetate ion acts only as a weak base. TMA in DMSO results in an irreversible transformation of the bilirubin; thus, with TMA, only the 2:1 Zn(II):bilirubin systems could be examined. During titration with TMA, the 455 nm (ϵ = 60,000 mol^{-1} cm^{-1} dm^3) absorption band of bilirubin shifted to 527 nm (ϵ = 69,000 mol^{-1} cm^{-1} dm^3), the orange-yellow solution becoming red. The number of equivalents of TMA suggests that the zinc(II) ions also bind OH groups during the addition of TMA, and Na-phenolate too was therefore applied as proton-binder to clarify the stoichiometric relations. The monofunctional phenolate ligand does not disturb the complex formation between zinc(II) and bilirubin, while at the same time it is a stronger proton-binder than acetate. Figure 1 shows the absorbance at 527 nm in the course of the titration of bilirubin with Na-phenolate.

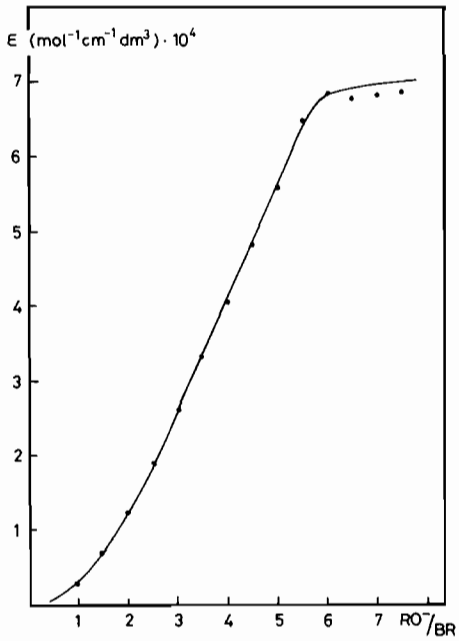


Fig. 1. Variation of molar absorbance of 2:1 zinc(II)–bilirubin solutions at 527 nm during titration with Na-phenolate. $c_{BR} = 3 \times 10^{-4}$ mol dm^{-3} in DMSO.

Figure 1 reveals that there is no further spectral change after the addition of 6 equivalents of phenolate, *i.e.* the interaction can be interpreted as in the following equation:

$$2Zn^{2+} + H_6BR + 6OR^- \rightleftharpoons 6ROH + [Zn_2BR]^{2-} \quad (1)$$

The formation of 2:1 complexes is supported by the fact that, when the 1:6 to 1:10 BR:Na-phenolate systems are titrated with Zn(II) ion, the same spectrum as the previous one is obtained after the addition of 2 equivalents of Zn(II), and the excess of metal ion having no subsequent effect.

In the Cd(II)–bilirubin system, the spectral changes and the composition of the complexes are perfectly analogous to those for the Zn(II)–bilirubin system. The absorption maximum for the complex Cd_2BR lies at 535 nm. In an inert atmosphere, the reactions are reversible for both zinc(II) and cadmium(II), *i.e.* the spectrum of bilirubin is restored if acid equivalent to the Na-phenolate is added to the complexes.

The biliverdin complexes were examined in metal ion–BV–TMA solutions with various compositions. TMA does not cause an irreversible change in the spectrum of free biliverdin, and it can therefore conveniently be applied as a strong proton-binder. In contrast with the results obtained for bilirubin, the two metal ions exhibited differing behaviour with biliverdin. Figure 2 presents the absorption spectra of Zn(II)–BV and Cd(II)–BV solutions of various compositions in DMSO.

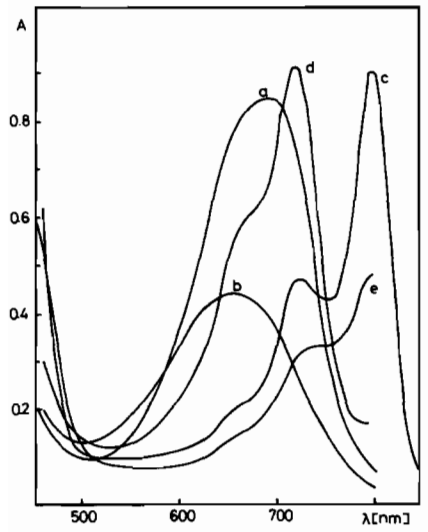


Fig. 2. Spectra of biliverdin and its zinc(II) and cadmium(II) complexes in DMSO. $c_{BV} = 3 \times 10^{-4}$ mol dm^{-3}. (a) BV·2HCl; (b) BV·2HCl + 4 equivalents of TMA; (c) Cd:BV:TMA = 1:1:8; (d) Zn:BV:TMA = 1:1:8; (e) Zn:BV:TMA ; 1:1:30.

Figure 2 demonstrates that the deprotonation of BV·2HCl (spectrum **a**) results in a slight blue shift of the visible absorption band and a decrease in the absorbance. Spectrum **b** relates to a sample containing 4 equivalents of TMA; the excess of TMA does not cause any further spectral change. If zinc(II) or cadmium(II) is added to a solution containing BV·2HCl, then, similarly as for bilirubin, a spectral change is not observed. The complex formation becomes appreciable only in the presence of proton-binding substances. Spectrum **c** shows the characteristic absorption curve for the 1:1 Cd(II):BV solution after the addition of 8 equivalents of TMA; this is indicative of the following reaction:

$$Cd^{2+} + H_6BV \cdot 2HCl + 8OH^- \rightleftharpoons 8HOH + 2Cl^- + [CdBV] \quad (2)$$

Equation 2 finds support from the fact that neither a TMA nor a Cd^{2+} excess results in a further spectral change. Accordingly, biliverdin, which contains a conjugated double bond system, is more prone to form 1:1 complexes.

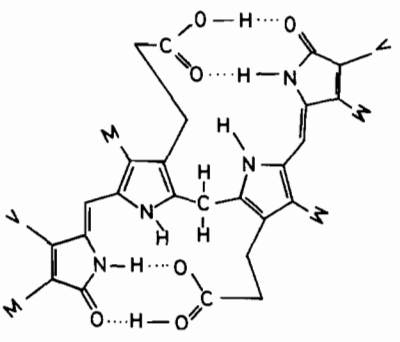
It is substantially more difficult to interpret our results for the zinc(II)–biliverdin system. Spectrum **2d** relates to a solution with the same concentration relations as for **2c**, but also containing Zn^{2+}. Since an excess of Zn^{2+} does not cause a further change, it might be concluded from the slight, but likewise red shift that a reaction analogous to that in eqn. 2 occurs. However, this is contradicted by the observation that in both 1:1 and 2:1 Zn(II)–BV solutions a TMA excess causes a further red shift; the spectrum becomes constant only after the addition of 30 equivalents of TMA (spectrum **2e**). As BV does not contain more dissociable protons, and spectrum **2e** approximates well to that for the Zn(II)–BV complex in aqueous medium (spectrum **4b**), it may be assumed that the spectral change is due to the conformation change caused by the formation of mixed hydroxo complexes. It should be noted that the complex formation is 'reversible' for both metal ions, *i.e.* the spectrum of BV·2HCl is obtained if acid is added to solutions **2c**, **d** and **e**. The reversibility is complete in an inert atmosphere, but even aerobic conditions do not result in a considerable degree of oxidation.

2. Complexes of Zinc(II) and Cadmium(II) in Aqueous Solution

As mentioned in connection with the examinations in DMSO, the metal ion–bilirubin interaction was not influenced significantly by the presence of air or by its exclusion. At the same time, the processes occurring in aqueous medium differ fundamentally in the presence or in the absence of O_2.

When an equivalent amount of zinc(II) or cadmium(II) was added to a bilirubin solution at pH 10.5 in an inert atmosphere, no change was observed. This indicates, in contrast with the earlier reports [4, 5], that complex formation does not take place between bilirubin and zinc(II) or cadmium(II) ions. This means that the pH of the solutions is not high enough for the metal ion-induced deprotonation of the pyrrole NH groups (with pK > 14) to occur. This result is not surprising, for the zinc(II) ion is not able to substitute the NH hydrogen atom in other compounds containing a pyrrole NH group [9]. The difference between the results in aqueous and non-aqueous media may presumably be interpreted by the change in conformation of the bilirubin. The NMR study by Kuenzle *et al.* [10] suggests that in non-aqueous medium bilirubin has structure **I**, *i.e.* the arrangement of the bonding sites is determined by intramolecular H-bonds.

Water as solvent, however, breaks down these internal H-bonds, and this eliminates the arrangement



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of the donor atoms which permits the (N,N,O)-coordination proposed by Van Norman and Szentirmay [7] in the complex Zn_2BR.

However, if the interaction is examined under aerobic conditions (even traces of O_2 are sufficient for dilute solutions), a continuous change of the bilirubin spectrum may be observed in the presence of the metal ions. In the course of this, the bilirubin absorption maximum at 435 nm progressively shifts to lower wavelengths, and the intensity decreases. Further, new low-intensity absorption bands appear in the visible region, which indicates that compounds containing conjugated double bonds may be present; these are presumably various bilirubin oxidation products according to the Gmelin series [11]. They are illustrated in Fig. 3.

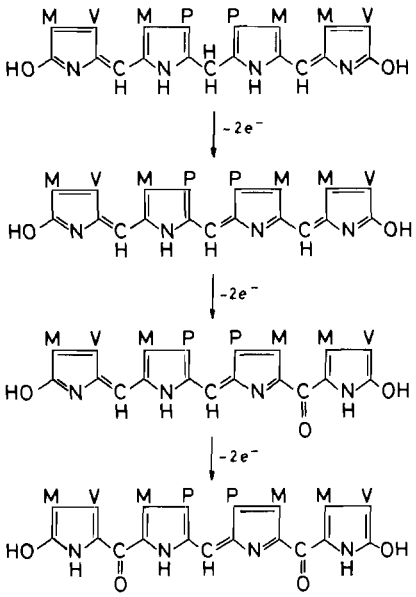

Fig. 3. Oxidation products of bilirubin (bilirubin → biliverdin → purpurin → choletelin) (M = methyl, P = propionic acid, V = vinyl).

Thus, zinc(II) and cadmium(II) ions 'catalyze' the oxidation of bilirubin, a rather surprising process for these metal ions. The phenomenon becomes understandable, however, if consideration is paid to the possible interactions between the metal ions and biliverdin, an oxidation product of bilirubin.

Spectra recorded during the reactions of zinc(II) and cadmium(II) ions with biliverdin in an inert atmosphere are presented in Fig. 4.

Spectrum **a** relates to free biliverdin, while spectra **b** and **c** were obtained after the addition of one equivalent of zinc(II) and cadmium(II), respectively. The spectral changes are similar to those observed in DMSO medium (spectra **2c** and **2e**), *i.e.* 1:1 complexes are formed in aqueous medium too. Accordingly, in contrast with bilirubin, in the case of

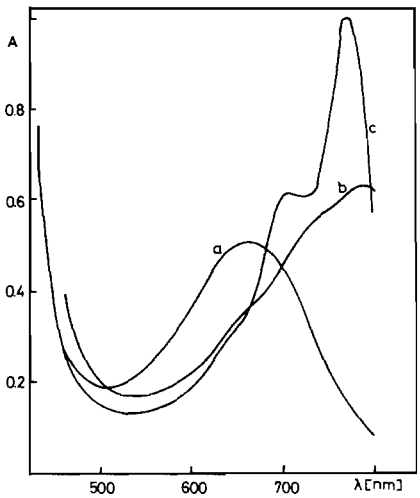

Fig. 4. Spectra of biliverdin and its complexes in aqueous medium at pH 10.5. $c_{BV} = 3 \times 10^{-4}$ mol dm^{-3}. (a) BV; (b) Zn(II):BV = 1:1; (c) Cd(II):BV = 1:1.

biliverdin (containing conjugated double bonds) zinc(II) and cadmium(II) ions are able to replace the pyrrole hydrogen atoms, and thus a stable triple chelate system may develop. On the action of excess EDTA, the initial BV spectrum is recovered in both cases, which is evidence that the changes arise not from oxidation of the ligand, but from complex formation. If the pH of the solutions is decreased to 8.5 with hydrochloric acid, the spectrum of BV is again obtained, as an indication that complex formation occurs in the pH interval 8.5–10.5. On this basis, if it is taken into account that biliverdin has the higher donor strength, it is not surprising that there is no complex formation with bilirubin up to pH 10.5.

If the 1:1 solutions are left to stand in air, further spectral changes occur very rapidly, as illustrated in Fig. 5.

Spectrum **5a** obtained with cadmium(II) as central ion. If excess EDTA is added to this solution, the colour becomes red; the resulting spectrum is that in Fig. **5b**. Since spectrum **5b** and the colour of the solution are characteristic of those for purpurin, it is probable that in the presence of O_2 biliverdin undergoes oxidation and purpurin complexes are formed. Spectrum **5a** is not constant in time, *i.e.* the fast oxidation to purpurin may be followed by further oxidation steps according to the Gmelin series, in a slower process.

The spectral characterisitics suggest that spectrum **5c** may be identified with the Zn(II)–purpurin complex. However, the addition of EDTA does not cause a spectral change in this case, which indicates that the small zinc(II) ion forms a complex of high stability. Such a high stability might explain the fast oxidative transformation too.

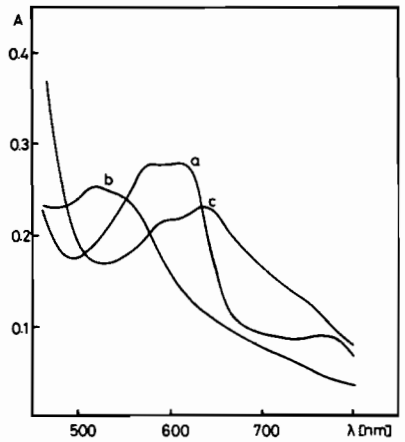

Fig. 5. Spectra of products formed in the zinc(II)–biliverdin and cadmium(II)–biliverdin systems at pH 10.5 on the action of oxygen. (a) $c_{Cd} = c_{BV} = 3 \times 10^{-4}$ mol dm^{-3}; (b) solution (a) + excess EDTA; (c) $c_{Zn} = c_{BV} = 3 \times 10^{-4}$ mol dm^{-3}.

These results lend support to our observation that the metal ions accelerate the oxidative transformation of bilirubin. Naturally, the accelerating effect does not occur through direct activation of the O_2 molecule; the metal ions rather form stable complexes with the small quantities of oxidation product always present, thereby shifting the transformation in the direction bilirubin → biliverdin → purpurin.

3. Copper(II) Complexes of Bilirubin and Biliverdin

When bilirubin is mixed with copper(II) ion in DMSO medium, the orange-yellow solution becomes green within a relatively short time, even in an inert atmosphere. The typical spectral changes are depicted in Fig. 6.

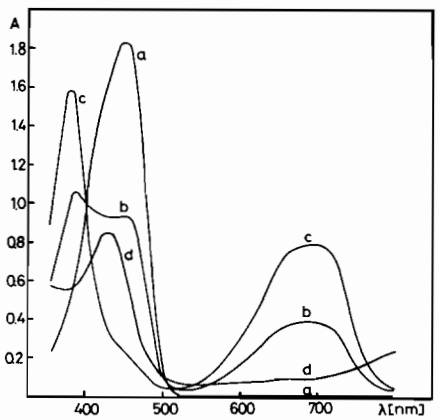

Fig. 6. Spectra relating to interactions of copper(II) with bilirubin or biliverdin in DMSO. $c_{BR} = 3 \times 10^{-4}$ mol dm^{-3}. (a) BR; (b) BR:Cu(II) = 1:1; (c) BR:Cu(II) = 1:2; (d) BV:Cu(II):TMA = 1:1:8.

Spectrum **6a** is due to bilirubin, while spectra b and **c** were obtained after the addition of 1 and 2 equivalents of copper(II), respectively. Since spectrum **6c** is practically identical with the spectrum of BV·2HCl, it seems reasonable to assume that the following redox process takes place:

$$2Cu^{2+} + BR = BV + 2Cu^{+} + 2H^{+} \qquad (3)$$

This assumption is supported by the fact that solutions **6b** and **6c** are both stable in N_2 atmosphere. If O_2 is introduced to solution **6b**, however, the oxidation of the copper(I) leads to the resumption of the redox reaction in eqn. 3; this proceeds fairly slowly, but quantitatively. In accordance with this assumption, if copper(II) ion is added to biliverdin, no spectral change can be observed. In non-aqueous medium, therefore, the copper(II) ion is not able to replace the NH protons of either bilirubin or biliverdin, and (similarly as for zinc(II) and cadmium(II) ions) complex formation is to be expected only in the presence of proton-binding substances. Spectrum **6d** relates to the copper(II)–biliverdin–TMA interaction. Complex formation shifts both the visible and the UV absorption bands to longer wavelengths. Studies at various BV/Cu(II) ratios reveal that, similarly as for zinc(II) and cadmium(II), a 1:1 complex is formed in an analogous way as in eqn. 2.

Investigations in aqueous medium led to results conforming with the above, but the oxidative stability of the products formed is essentially lower. When biliverdin is reacted with copper(II) ion in buffer of pH 10.5 in an inert atmosphere, the ratio-dependence points to the formation of a 1:1 complex. However, the oxidative stability of this complex is extremely low, and on the action of air it is very readily converted to an other product which can presumably be assigned to copper(II)–purpurin. Similarly as for zinc, but in contrast with cadmium, the reaction can not be reversed by the addition of EDTA, *i.e.* the purpurin can not be demonstrated directly, because of the high stability of the complex.

In contrast with the zinc(II) and cadmium(II) ions, when the reaction between copper(II) ion and bilirubin is examined in aqueous medium a rapid change of the bilirubin spectrum is observed under all conditions. This change is a result of the redox reaction as in eqn. 3. In this case, however, the biliverdin formed can not be detected, as it immediately forms a complex with the metal ion excess. Accordingly, the interaction of bilirubin and copper(II) in aqueous medium in an inert atmosphere yields a light-brown biliverdin complex, while under aerobic conditions a dark-green purpurin complex is obtained.

Conclusions

Figure 7 shows the possible complex formation and redox reactions that are assumed to take place between the metal ions and bilirubin and biliverdin on the basis of the above experimental results.

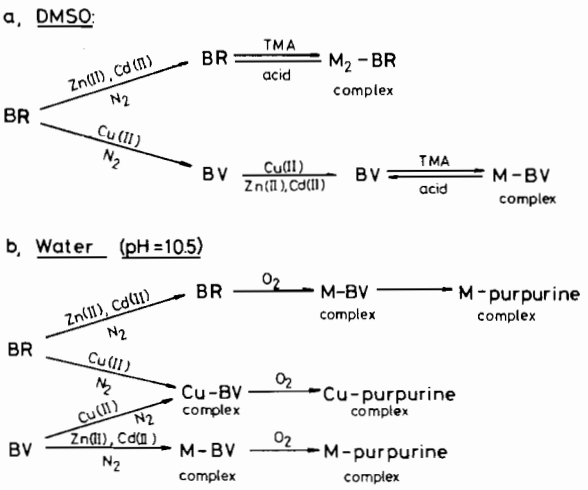

Fig. 7. Possible pathways of interaction of bilirubin and biliverdin with metal ions.

The Figure indicates that bilirubin does not interact directly with zinc(II) and cadmium(II) ions in either DMSO or aqueous medium, *i.e.* these metal ions are not able to induce the ionization of the pyrrole NH. In the presence of bases (CH_3COO^-, RO^-, OH^-) in DMSO, however, this reaction can occur and 2:1 complexes are formed. Under all conditions, the copper(II) ion results in the relatively fast oxidation of bilirubin, biliverdin being obtained in DMSO, and a copper(II)–biliverdin complex in aqueous medium.

In accordance with this, in aqueous medium biliverdin forms 1:1 complexes with all three metal ions, while in DMSO these processes take place only in the presence of proton-binding substances. It may be stated further that the complexes formed in DMSO are comparatively stable even in air, whereas in aqueous medium all three metal ions considerably accelerate the oxidative transformation of bilirubin. In the case of copper(II), this takes the form of a redox reaction as in eqn. 3, together with the interaction of the biliverdin complexes with O_2. With the zinc(II) or cadmium(II) ion as central ion the direct redox reaction is not possible, and the metal ions therefore accelerate the reactions of bilirubin and biliverdin with O_2 via the formation of stable M(II)–biliverdin and M(II)–purpurin complexes.

Thus, the interpretation of the complex-forming properties of bilirubin and biliverdin necessitates a many-sided approach to the study of the metal ion–ligand interaction. As pointed out previously [6, 7], the acid–base properties of bilirubin and biliverdin must be taken into account. However, it is also important to consider the direct redox reactions with the various metal ions, and the oxidation processes with O_2 in the presence of metal ions.

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