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On the Molecularity of Bilirubins and their Esters and Anions in Chloroform Solution

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Summary. Bilirubins with propionic acids at C-8 and C-12 engage in intramolecular hydrogen bonding and are thought to be monomeric in solution, although the latter is unproven. In contrast, their dimethyl esters and etiobilirubin analogs (with the C-8 and C-12 propionic acids replaced by alkyl residues) favor intermolecular hydrogen bonding and are thought to be dimeric in nonpolar solvents. There is little information on the molecularity of the bilirubin dianion in solution. In this work, vapor pressure osmometry studies of chloroform solutions of bilirubins, their dimethyl esters, and etio-analogs clearly indicate that the diacids and dianions are monomeric, whereas the diesters and dialkyls are dimeric. However, the presence of a C-10 *gem*-dimethyl group causes the ester and the etiobilirubin to become monomeric.

Keywords. Bile pigments; Solution molecular weight; Aggregation; Vapor pressure osmometry.

Introduction

Bilirubin (Fig. 1) is a festuscine-colored tetrapyrrole discarboxylic acid that is produced in vertebrates, including humans, principally from the breakdown of red blood cells [1, 2]. Although intrinsically unexcretable, it is efficiently eliminated by the liver following uptake and enzymic conversion to water-soluble glucuronides that are promptly secreted into bile. Impaired excretion of the glucuronides occurs in many types of hepatobiliary disease, but retention of native bilirubin is observed principally in newborn babies [1–3]. Accumulation of either native bilirubin or its glucuronides in the body is manifested by jaundice.

Bilirubin, one of the most interesting natural product members of the linear tetrapyrrole class [4], is a conformationally mobile bichromophore with characteristics of a molecular propeller. Rotation of its two dipyrrinone chromophores about the central CH_2 unit generates a large number of conformational isomers, of which only the ridge-tile shaped, folded conformations have their non-bonded steric interactions minimized [5]. The ridge-tile conformation brings the pigment's propionic acid groups into close proximity with the dipyrrinone NH and C=O groups where they easily embrace to form a network of six intramolecular hydrogen bonds (Fig. 1). This inward tucking of the CO_2H groups and their tethering to

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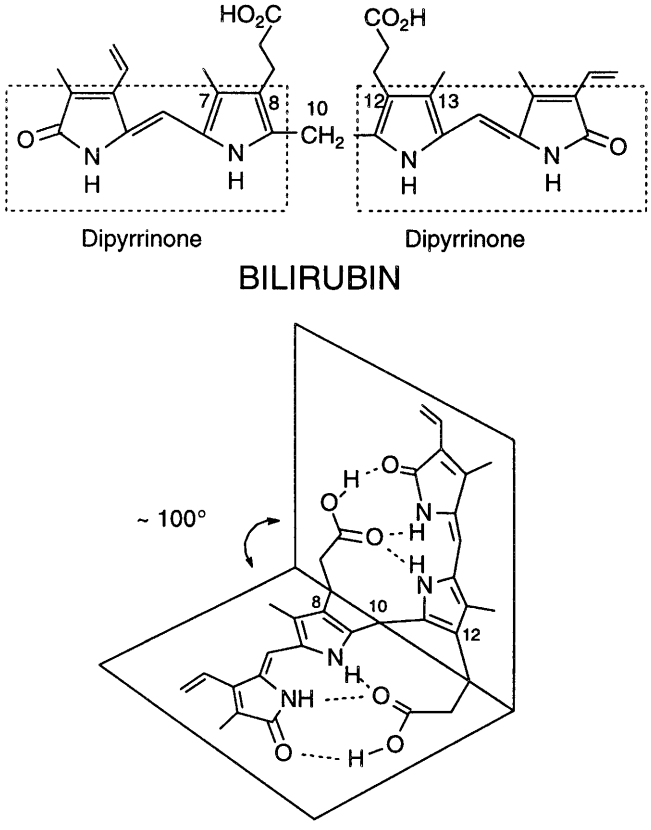


Fig. 1. (Upper) Bilirubin displayed in a linear conformation and showing its two dipyrrinone components; (lower) ridge-tile conformation of bilirubin stabilized by intramolecular hydrogen bonding between the dipyrrinones and propionic acids; hydrogen bonds are indicated by dashed lines

opposing dipyrrinones through intramolecular hydrogen bonding makes the ridge-tile conformation unusually stable [3–6]. Such hydrogen bonding serves to decrease the polarity of the pigment, leaving it unexcretable in normal hepatic metabolism, except by glucuronidation [1–3].

The ridge-tile conformation is found in crystalline bilirubin [7–9], and it is the favored conformation in solution [5, 10, 11]. The intramolecular hydrogen bonding so essential to preserving the conformation is driven by the strong disposition of its constituent dipyrrinones toward forming hydrogen bonds [5, 12, 13]. Even simple dipyrrinones such as xanthobilirubic acid and its homologs form dimers that utilize the dipyrrinone-carboxylic acid hydrogen bonding [14, 15] (Fig. 2) characteristic of that found in bilirubin (Fig. 1) [5]. However, dipyrrinone esters such as methyl xanthobilirubinate form planar, hydrogen-bonded dipyrrinone-dipyrrinone dimers, as do dipyrrinones with alkyl β-substituents, such as kryptopyrromethenone (Fig. 2) [4, 12, 13]. Dipyrrinone-dipyrrinone intermolecular hydrogen bonding is also thought to be important in bilirubin dimethyl ester, which forms a dimer in nonpolar solvents [4, 16–20].

Although bilirubin is assumed to be monomeric in nonpolar solvents, its solution molecular weight has not been reported, even in chloroform, the nonpolar

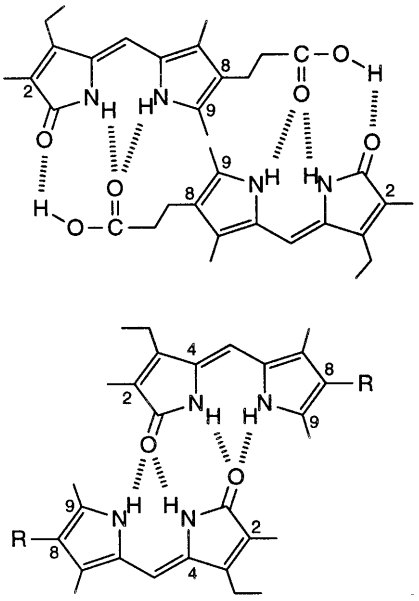


Fig. 2. (Upper) Xanthobilirubic acid dimer with acid-to-dipyrrinone hydrogen bonding as found in bilirubin and its analogs with propionic acids at C-8 and/or C-12; this dimer is not planar; the two dipyrrinones lie in parallel planes (Ref. 15); (lower) planar dipyrrinone-to-dipyrrinone hydrogen bonding found in bilirubin esters, rubins incapable of intramolecular hydrogen bonding, and in many 4(*Z*)-dipyrrinones, such as kryptopyrromethenone ($R = CH_2CH_3$) and methyl xanthobilirubinate ($R = CH_2CH_2CO_2CH_3$); hydrogen bonds are shown by hatched lines

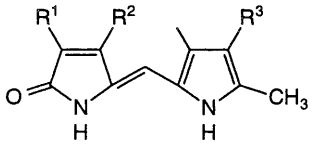
solvent in which it is most soluble (~ 1 m*M*) [21]. The bilirubin dianion is thought to be intramolecularly hydrogen-bonded [22–24], but whether it is monomeric in solutions has not been determined. Our interest in the role of dipyrrinones and carboxyl groups in a monomer-dimer equilibrium of bilirubins as well as their dianions and diesters led us to determine the state of aggregation of chloroform soluble bilirubins, dimethyl esters, dianions, and etiobilirubins.

The current investigation uses vapor pressure osmometry (VPO) measurements of fourteen related rubins and presents new information on the state of aggregation and the monomer-dimer equilibrium of bilirubins, diesters, and dianions in the hydrogen bond-promoting, nonpolar solvent chloroform. This comprehensive study reports the first systematic investigation of the molecularity of bilirubin diacids, diesters, and dianions.

Results and Discussion

The apparent molecular weights of only a few dipyrrinones and bilirubins in solution have been determined. VPO measurements by *Falk et al.* [4, 13] of methyl xanthobilirubinate and kryptopyrromethenone (Fig. 2) in chloroform revealed dimer formation ($K_{\text{dimer}} \sim 1700\,M^{-1}$ at 37°C), although the actual measured molecular weights were not specifically indicated. More recent studies involving ^{1}H NMR analyses of the concentration dependence of the NH chemical shifts confirm that

Table 1. Molecular weights of dipyrrinones and bilirubin analogs determined by vapor pressure osmometry[a]



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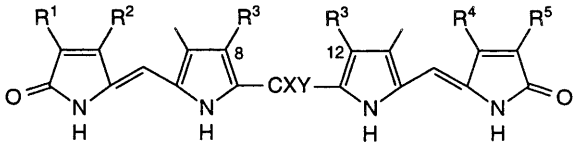

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Entry		*X*	*Y*	R^1	R^2	R^3	R^4	R^5	Formula Weight	Measured Molecular Weight
1[b,c]	**D**	–	–	*M*	*E*	*E*	–	–	258.3	509
2[c,d]	**D**	–	–	*M*	*E*	*PM*	–	–	316.3	579
3[e]	**T**	H	H	*M*	*E*	*E*	*E*	*M*	500.7	800, 966[e]
4[e]	**T**	H	H	*M*	*V*	*PM*	*M*	*V*	612.7	1173, 850[e], 1100[f]
5[g]	**T**	H	H	*M*	*E*	*PM*	*E*	*M*	616.7	1139, 1250[g]
6[h]	**T**	H	H	*B*	*M*	*P*	*M*	*B*	644.8	632
7[h]	**T**	H	H	*M*	*B*	*P*	*B*	*M*	644.8	637
8[i]	**T**	*M*	*M*	*M*	*M*	*P*	*M*	*M*	588.7	593
9[i]	**T**	*M*	*M*	*M*	*M*	*PM*	*M*	*M*	616.7	610
10[j]	**T**	H	*M*	*E*	*E*	*PM*	*E*	*E*	658.8	1173
11[k]	**T**	H	*tB*	*M*	*M*	*PM*	*M*	*M*	644.9	1272
12[j]	**T**	H	*M*	*E*	*E*	*P*	*E*	*E*	630.7	629
13[k]	**T**	H	*tB*	*M*	*M*	*P*	*M*	*M*	616.8	649
14[l]	**T**	H	*A*	*M*	*M*	*M*	*M*	*M*	578.8	1158
15[m]	**T**	H	H	*M*	*V*	P^-	*M*	*V*	1067.6	617[n]
16[m]	**T**	H	H	*M*	*E*	P^-	*E*	*M*	1071.6	611[o]

[a] Formula weights and measured molecular weights in g/mol; measured molecular weights ±5% for 1.5–6.5 m*M* solutions in $CHCl_3$ at 45°C; *M* = methyl, *E* = ethyl, *V* = vinyl, *B* = *n*-butyl, *tB* = *tert*-Butyl, *A* = l-adamantyl, *P* = propionic acid, *PM* = methyl propionate, P^- = propionate salt with tetra-*n*-butylammonium ion; [b] Ref. [13]; [c] Refs. [4] and [13] report that this dipyrrinone is dimeric in chloroform, $K_{dimer} \sim 1700\,M^{-1}$; [d] Refs. [13] and Shrout DP, Lightner DA (1990) Synthesis 1062; [e] Refs. [4, 16]; [f] Ref. [18] reports this tetrapyrrole to be a dimer; [g] Ref. [25]; [h] Ref. [26]; [i] Ref. [27]; [j] Kar A (1998) PhD Thesis, University of Nevada; [k] Kar A, Lightner DA (1998) Tetrahedron **54**: 12671; [l] Kar A, Lightner DA (1998) J Heterocyclic Chem **35**: 795; [m] Refs. [22, 31, 32]; [n] Formula weight of naked dianion: 582.7 g/mol; [o] Formula weight of naked dianion: 586.7 g/mol

these dipyrrinones favor a dimer, with values of K_{dimer} of about $25000\,M^{-1}$ at 25°C in chloroform [12]. The studies clearly indicated an NOE between C(2)-CH_3 and C(9)-CH_3, thus confirming the nonbonded spatial proximity of the dipyrrinones as in the dimeric structure of Fig. 2. Again using VPO, *Falk et al.* [4, 16, 17] determined the molecular weight of bilirubin dimethyl ester (formula weight 612.7 g/mol) to be 850±20 in chloroform and 1110±30 in *THF* at concentrations of 2×10^{-2} to 2×10^{-3} mol/kg. The preference for a dimer was confirmed independently by *Schaffner et al.* [18] and by *Kaplan* and *Navon* [19]. The related tetrapyrrole, with propionate esters replaced by ethyls, etiobilirubin-IVγ (formula weight 500.7 g/mol) was determined to have a molecular weight of 966±25 at

concentrations from 7×10^{-3} to 3×10^{-4} mol/kg in chloroform [4, 16]. More recently, *Ribó et al*. [25] showed by VPO that the dimethyl esters of mesobilirubin-IXα and -XIIIα (formula weight 616.7 g/mol) exhibited molecular weights corresponding to dimers ($\sim$ 950 and $\sim$ 1250, respectively).

In order to calibrate our VPO studies, we redetermined the molecular weights of kryptopyrromethenone, methyl xanthobilirubinate, etiobilirubin-IVγ, bilirubin dimethyl ester, and mesobilirubin-XIIIα dimethyl ester in chloroform (Table 1, entries 1–5) and found good agreement with the previous results that showed a strong preference for dimers.

Bilirubin is not appreciably soluble in nonpolar solvents [21], and although it is most soluble in chloroform, its molecular weight has not been reported in this solvent due to its limited high end solubility ($\sim$ 1 m*M*). In order to improve the solubility of bilirubin while preserving its ability to engage in intramolecular hydrogen bonding, we synthesized bilirubin analogs with vinyls replaced by *n*-butyls (one *n*-butyl group on each lactam ring – either both *exo* or both *endo*) [26] and determined by VPO that they were monomeric in chloroform (Table 1, entries 6 and 7). Similarly, we determined that a different synthetic analog of bilirubin, with a *gem*-dimethyl group at C-10 [27], is monomeric in chloroform (Table 1, entry 8), in accordance with ^{1}H NMR studies showing that it is intramolecularly hydrogen-bonded [28]. The data are consistent with and support the belief that bilirubin and its analogs which are capable of intramolecular hydrogen bonding are monomeric in chloroform.

The presence of propionic acid groups at C-8 and C-12 has been shown to have a dominating influence on bilirubin conformation by acting to preserve the ridge-tile conformation through intramolecular hydrogen bonding to the dipyrrinones (Fig. 1) [5]. This eumorphous and important conformation was deduced from ^{1}H NMR studies in chloroform solvent, including ^{1}H$\{^1$H$\}$ and ^{1}H$\{^{13}$C$\}$ NOE measurements [10, 11]. Since the rubin acids of Table 1 are known to engage in intramolecular hydrogen bonding and believed to adopt a ridge-tile conformation [26–28], and the VPO results clearly indicate that they are monomeric in solution, one might generalize to predict that when intramolecular hydrogen-bonding is present, monomers will be preferred in chloroform.

Absent such intramolecular hydrogen bonding to carboxylic acids, the dipyrrinones latch on to other dipyrrinones *via* intermolecular hydrogen bonding of the type shown in Fig. 2. Even though such concatenations might lead to polymeric arrays [29], bilirubin dimethyl ester is found to be only dimeric in chloroform [4, 16–19] (Table 1, entry 4), as is its close analog, mesobilirubin-XIIIα dimethyl ester [25] (Table 1, entry 5). Although propionate ester groups acting independently are insufficient to sequester the dipyrrinones through intramolecular hydrogen bonding, the pigment's conformation seems to be more amenable to dimer as opposed to polymer formation [29]. Consistent with this behavior, when the propionic esters are replaced with ethyl groups, the pigment is also dimeric (Table 1, entry 3), *e.g*. etiobilirubin-IVγ [4, 16]. Unexpectedly, however, when a C-10 *gem*-dimethyl group is present, the dimethyl ester is monomeric (Table 1, entry 9). Thus, whereas *gem*-dimethyl at C-10 does not interfere with the predisposition of rubin acids to be monomeric (and intramolecularly hydrogen bonded [28]) in chloroform, it has a profound effect on the dimethyl ester.

In order to explore further the influence of C-10 substituents on disfavoring the intermolecularly hydrogen-bonded dimerization of bilirubin esters, we determined that the molecular weight in chloroform of a bilirubin analog with only one C-10 methyl (Table 1, entry 10) corresponds to that of a dimer. Thus, for reasons as yet unclear, whereas one C-10 methyl is insufficient to disfavor dimer formation of bilirubin dimethyl ester, two C-10 methyls prevent dimer formation. Possibly the nonbonded steric interaction imposed on the C-2 methyl by the C-10 $CH(CH_3)$ group can be accommodated, whereas that by a C-10 $C(CH_3)_2$ is too large. However, even a C-10-*tert*-butyl is insufficient to prevent dimer formation in the dimethyl ester (Table 1, entry 11). As expected, the presence of but one C-10 methyl or *tert*-butyl has little effect on the state of aggregation of the corresponding bilirubin acid, as both are found to be monomeric (Table 1, entries 12 and 13).

There have been no reports of VPO measurements of the bilirubin dianion [30], and thus no information is available on its molecularity in solution. Previous studies involving ^{1}H NMR and circular dichroism spectroscopy [22, 23, 31, 32] indicate that when the propionic acids are ionized, there is sufficient intramolecular hydrogen bonding to maintain a ridge-tile conformation. Even with two fewer hydrogen bonds, the remaining four are apparently intensified when the carboxylate anions engage in hydrogen bonding with the dipyrrinones. Our VPO results clearly show that both bilirubin and mesobilirubin-XIIIα dianions (Table 3, entries 15 and 16) are monomeric. Interestingly, these *bis*-tetra-*n*-butyl-ammonium salts exhibit molecular weights corresponding to the propionate anions freed of their tetra-*n*-butylammonium cations. The data are consistent with ^{1}H NMR chemical shift analyses [22, 32], ^{1}H{^{13}C} NOE studies [23], and even X-ray crystallography [30], all of which clearly indicate that the dianionic pigments are intramolecularly hydrogen-bonded.

Experimental

Vapor pressure osmometry measurements were performed on an OSMOMAT 070-SA instrument (Gonotec GmbH, Germany) in chloroform (Allied Signal, Burdick & Jackson, HPLC grade with amylene preservative, treated with activated basic alumina just before use) at 45°C. The source of each pigment used is indicated in Table 1.

Acknowledgments

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