Short Communications

A Proposed Novel Structure for the Metal Chelates of Bilirubin

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We have accumulated evidence for a novel conformational structure of bilirubin that is maximally stabilized by two pairs of intramolecular hydrogen bonds (C. C. Kuenzle, M. H. Weibel, R. R. Pelloni & P. Hemmerich, unpublished work). Each pair of bonds links one of the two carboxyl groups to the contralateral pyrrolenone end ring, so that the hydrogen atoms are interposed between both oxygen atoms of the carboxyl group on the one hand and the oxygen and nitrogen atoms of the pyrrolenone ring on the other. This arrangement specifies a conformation that is characterized by two molecular planes embracing an angle of approx. 110° . Each plane is occupied by one of the chromophoric moieties of bilirubin, which are separated by a central methylene bridge.

With this conformation in mind we have re-investigated the structure of the metal chelates of bilirubin. Ever since their original description (Ó Carra, 1962), these have been tacitly assumed to have structures analogous to the metalloporphyrins. However, conclusive evidence in support of this notion has never been forwarded (Fog & Bugge-Asperheim, 1964; Van Roy & Heirwegh, 1965; Nichol & Morell, 1969; Velapoldi & Menis, 1971). Moreover, steric considerations must raise serious doubts as to the correctness of this assumption. Quite to the contrary, the hydrogen-bonded conformation proposed for bilirubin suggests a structure for its metal chelates that imposes minimal steric strain on the molecule. In this structure each of the two chromophoric moieties is visualized as having the potential to complex with one metal ion, with the latter being trigonally co-ordinated to one oxygen and two nitrogen atoms (Fig. 1). Additional ligands may extend from the molecular planes, yielding complexes with co-ordination numbers greater than 3.

We are now advancing some evidence in support of this structure by showing that each molecule of bilirubin can indeed accommodate two metal ions. This conclusion has been reached by spectrophotometric titration of bilirubin with samarium acetate

(Velapoldi & Menis, 1971). Calculations derived from these measurements have further revealed markedly differing stability constants for incorporation of the first and the second metal ion, indicating mutual dependence of the two processes.

Commercial bilirubin (Fluka A.G., Buchs SG, Switzerland) was purified by the method of Fog (1964) [Found: C, 67.3; H, 6.1; N, 9.8. Calc. for $C_{33}H_{36}N_4O_6$ (mol.wt. 584.65): C, 67.8; H, 6.2; N, 9.6%. ϵ_{454} in chloroform-ethanol (1:1, v/v), 56700. The preparation was found by t.l.c. to be essentially devoid of contamination by bilirubin isomers III α and XIII α (McDonagh & Assisi, 1971)]. Samarium acetate was purchased from K & K Laboratories Inc., Plainview, N.Y., U.S.A., and was shown by elemental analysis to have the composition $Sm(CH_3CO_2)_3 \cdot H_2O$ [Found: Sm, 43.7; C, 20.5; H, 3.2. Calc. for $C_6H_{11}O_7Sm$ (mol.wt. 345.45): Sm, 43.5; C, 20.8; H, 3.2%]. Spectrophotometric titrations in chloroform-ethanol (1:1, v/v) with a Zeiss PMQII instrument were carried out in quartz cuvettes of either 1 cm or 2 cm light-path, depending on the bilirubin concentration. Spectra of all solutions were recorded with a Beckman DB spectrophotometer, and were found to be qualitatively consistent with data in the literature (Velapoldi & Menis, 1971). In particular, the peak absorption of the complex occurred at 395 nm.

The low solubility of samarium acetate in organic solvents necessitated the performance of two sets of experiments: one in which the use of a relatively high concentration of bilirubin ($15.6 \mu M$) allowed the precise titration at low $[Sm^{3+}]/[bilirubin]$ ratios (up to 13:1), and another in which a low concentration of bilirubin ($4.44 \mu M$) facilitated the measurement over a broad range of molar ratios (up to 46:1). Bilirubin was dissolved in chloroform and was diluted with an equal volume of ethanol. Samples (50 ml) were pipetted into volumetric flasks and were made up to 100 ml with serial dilutions (15 to 18 in each experiment) of a stock solution of samarium acetate prepared by dissolving the compound in 1 ml of water and diluting to 1 litre with chloroform-

Fig. 1. Steric representation of the structure proposed for the metal chelates of bilirubin

The compound is suggested to have the potential to complex with two metal equivalents, each of which may be accommodated by one of the two chromophores. The metal ions (M) would be co-ordinated in a trigonal arrangement to two nitrogen atoms and one oxygen atom. Additional ligands (L) may extend normal to the molecular planes defined by the two chromophores, but their exact number and orientation presently must remain unspecified. It should be noted, however, that any such ligands would exhibit mutual steric repulsion. For reasons of clarity the various bond types have not been drawn differently.

ethanol. The solutions were allowed to reach equilibrium overnight at room temperature in the dark (Velapoldi & Menis, 1971) before readings were taken at 395nm. To correct for oxidative decomposition of bilirubin during equilibration, a solution of bilirubin containing no Sm^{3+} was measured at 454nm immediately after preparation and again at the time of spectrophotometric titration. The decay of absorbance was taken as a measure of bilirubin degradation, and the assumption was made that the rates of destruction were identical for complexed and uncomplexed bilirubin. Decomposition ranged from 8.9 to 23.6%.

Fig. 2 shows the titration curve of low-concentration bilirubin with samarium acetate. A qualitatively similar plot was obtained with bilirubin at the higher concentration specified. A prominent feature of the plot was its diphasic course, exhibiting a first rise at $[\text{Sm}^{3+}]/[\text{bilirubin}]$ ratios lower than 4 followed by a second rise above this limit. The simultaneously recorded optical spectra gave evidence of a gradual shift of the absorption maximum from 454nm (uncomplexed bilirubin) to 395nm (attained at a $[\text{Sm}^{3+}]/[\text{bilirubin}]$ ratio of approx. 9) followed by a continuously increasing preponderance of the absorbance at 395nm over the long-wavelength band now shifting

to approx. 470nm. This indicated that the two-step process revealed by the titration plot resulted from changes that were qualitatively similar but differed in the ease with which they occurred. This behaviour is exactly what one would expect of a system as outlined in Fig. 1, in which the complexing of a first and later of a second metal ion/molecule of bilirubin ultimately yields two identically altered chromophores, whereas at intermediate molar ratios of the reactants uncomplexed and complexed chromophores would contribute differently to the overall spectrum observed. The fact that the second metal equivalent enters the complex less readily than the first, as revealed by the high Sm^{3+} concentrations needed for this step, is indicative of a mutual dependence of the two complex-formation stages and may be viewed as arising from steric interaction between additional ligands appended to the metallic centres as shown in Fig. 1. Ligands extending to the inner of the pleated-sheet structure may generate mutual steric repulsion, rendering the second complex-formation stage energetically less favourable than the first unhindered one. Thus the experimental findings are consistent with the structure proposed for the complex.

The titration plots were also treated quantitatively in order to obtain an estimate of the stability con-

Fig. 2. Spectrophotometric titration plot obtained on titration of bilirubin ($4.44\mu\text{M}$) in chloroform-ethanol (1:1, v/v) with samarium(III) acetate

The curve's diphasic course should be noted. Absorbance values at 395 nm are given as pertaining to a light-path of 1 cm although the measurements were actually taken in 2 cm cuvettes.

stants defining the two complex-formation steps. The experiments performed with the higher and lower bilirubin starting concentrations served as the respective sources of data for the computation of stability constants K_1 and K_2 pertaining to the complexing of the first and the second metal ion respectively. Theoretically both steps should conform to hyperbolic functions. However, analysis of the first rise of the plot was complicated by the fact that in this region three molecular species contribute differentially to the absorbance at 395 nm, i.e. uncomplexed bilirubin and bilirubin complexed with one and with two metal ions. The first step was better approximated by an exponential function, and this served to statistically average the experimental values. However, the efficacy of this approach may be questioned in view of the large standard deviation that had to be accepted with the final value of stability constant K_1 (see below). Mathematical treatment of the second step was much more effective in decreasing statistical spread, as evidenced by the small standard deviation of K_2 (see below), and this was attributed to the observation that, at least in its upper part, the experimental plot neatly fitted a hyperbolic function. The lower part of the plot defining the second stage of complex-formation was grossly distorted, owing to significant contributions from all of the three molecular species defined above. This part of the plot was therefore not used for calculations.

Both the exponential function approximating the first rise of the curve and the hyperbolic function defining the second stage yielded the statistically corrected absorbance values from which were calculated the various concentrations of bilirubin (B),

Sm^{3+} and the two bilirubin-samarium complexes containing one (B-Sm) and two (B-Sm₂) metal equivalents respectively. This was possible after extrapolation of the plots to zero and infinite Sm^{3+} concentrations, affording the molar extinction coefficients at 395 nm ϵ_{B} , 25000, $\epsilon_{\text{B-Sm}}$, 29500, and $\epsilon_{\text{B-Sm}_2}$, 75500. Calculated from:

$$K_1 = \frac{[\text{B-Sm}]}{[\text{B}][\text{Sm}^{3+}]} \quad (1)$$

corrected absorbance at 395 nm =

$$\epsilon_{\text{B}}[\text{B}] + \epsilon_{\text{B-Sm}}[\text{B-Sm}] \quad (2)$$

total bilirubin starting concentration =

$$[\text{B}] + [\text{B-Sm}] \quad (3)$$

total samarium starting concentration =

$$[\text{Sm}^{3+}] + [\text{B-Sm}] \quad (4)$$

in the mathematical treatment of the first complex-formation stage, and from:

$$K_2 = \frac{[\text{B-Sm}_2]}{[\text{B-Sm}][\text{Sm}^{3+}]} \quad (5)$$

corrected absorbance at 395 nm =

$$\epsilon_{\text{B-Sm}}[\text{B-Sm}] + \epsilon_{\text{B-Sm}_2}[\text{B-Sm}_2] \quad (6)$$

total bilirubin starting concentration =

$$[\text{B-Sm}] + [\text{B-Sm}_2] \quad (7)$$

total samarium starting concentration =

$$[\text{Sm}^{3+}] + [\text{B-Sm}] + 2[\text{B-Sm}_2] \quad (8)$$

with the second complex-formation stage, the values of the stability constants were obtained as:

$$K_1 = 3.3 \times 10^5 \pm 2.1 \times 10^5 \text{ (mean} \pm \text{s.d.)}$$

and

$$K_2 = 4.8 \times 10^4 \pm 0.3 \times 10^4 \text{ (mean} \pm \text{s.d.)}$$

Discrepancies found in three instances with the report of Velapoldi & Menis (1971) deserve a short mention. First, these authors have stated that oxidative degradation of the complex was negligible under their conditions of measurement. Although complex-formation requires 15 h for completion, thus complicating the determination of the exact rate of decomposition, measurements made by us in one instance have shown that degradation of the complex was significant and was comparable with that of uncomplexed bilirubin. This discrepancy might be related to the fact that the type of sophisticated equipment used by Velapoldi & Menis (1971) was not available in our laboratory, therefore prohibiting the continued storage under N_2 of the solutions to be measured. Secondly, we have not been able to confirm the previous report that a samarium/bilirubin ratio of 4:1 resulted in essentially 100% complex-formation. In our experience this molar ratio only suffices to bring the first complex-formation stage to completion. Therefore we suspect that previous spectrophotometric titrations have not been performed beyond this point, leaving the second rise of the titration plot undetected. Thirdly, the molar extinction coefficient of the bilirubin-samarium com-

plex has been given as 46400, whereas in our investigation the values determined for the two complex-formation steps were 29500 and 75700 respectively. Although the reason for this discrepancy is not entirely understood, we suggest that it may be partly due to the difficulty of obtaining accurate measurements from the somewhat arbitrary extrapolations of the titration plot to infinite Sm^{3+} concentrations.

Be that as it may, the absolute values of the quantitative data have no direct bearing on the argument put forward in the present investigation. Rather it is the qualitative aspects of the titration plots that are pertinent to this study. Indeed, the double rise of the curve affords strong evidence in support of the proposal that bilirubin has the potential to form complexes with two metal equivalents, thus strengthening the case for its suggested molecular structure.

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