Metal Complexes of Bilirubin in Aprotic Solvents

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Bilirubin reacts with zinc acetate, cadmium acetate or cobaltous acetate in dry dipolar aprotic solvents to form highly coloured complexes. The complex formed in *NN*-dimethylformamide can be isolated as a solid with bilirubin:zinc:acetate:*NN*-dimethylformamide molar proportions of 1:3:2:2. Water will decompose these complexes, with the regeneration of bilirubin.

Bilirubin inhibits some Zn^{2+} -requiring enzymes (Flitman & Worth, 1966; Yamaguchi, 1970) and Zn^{2+} may play an important part in liver function (Vallee, 1959). Complexes formed from bilirubin and Zn^{2+} or other metal ions have been observed in solution (O'Carra, 1962; Velapoldi & Menis, 1971), and a spectrophotometric investigation of bilirubin–samarium acetate complexes has been described (Kuenzle *et al.*, 1972). We now report the formation of stable complexes between bilirubin and zinc salts of aliphatic acids in dipolar aprotic solvents.

Experimental

Bilirubin, *NN*-dimethylformamide and dimethylsulphoxide were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Bilirubin was purified by the method of Fog (1964) before use (Found: C, 67.9; H, 6.0; N, 9.2; $\text{C}_{33}\text{H}_{36}\text{N}_4\text{O}_6$ requires C, 67.8; H, 6.2; N, 9.6%). *NN*-Dimethylformamide was dried over BaO and then distilled and redistilled *in vacuo* from 4A molecular sieves. Dimethyl sulphoxide was dried over CaH_2 and then distilled and redistilled as described above for *NN*-dimethylformamide. Both solvents were stored over 4A molecular sieves. Zinc [^3H]acetate (4.54 g) was prepared from ZnO (4.0 g) and a solution of [$\text{U-}^3\text{H}$]acetic acid (5 ml) in $^3\text{H}_2\text{O}$ (15 ml). Zinc [^{14}C]acetate was prepared by adding sodium [^{14}C]acetate (50 μCi) to unlabelled material (2 g), converting this into aqueous acetic acid by means of Dowex 50 (H^+ form) ion-exchange resin and then adding basic zinc carbonate. Concentration of the filtrate *in vacuo* gave crystalline zinc [^{14}C]acetate. The ^{14}C content of samples was measured by using dioxan-based scintillation medium as previously described (Hutchinson *et al.*, 1971).

Results

Isolation of bilirubin–zinc acetate complexes from NN-dimethylformamide. To bilirubin (100 mg, 0.17 mmol) in dry *NN*-dimethylformamide (150 ml) was added zinc acetate dihydrate (150 mg, 0.68 mmol)

in dry *NN*-dimethylformamide, and the deep-red solution was set aside for 10 min in the dark. *NN*-Dimethylformamide (approx. 130 ml) was removed *in vacuo* below 40°C, and the concentrate was set aside for 1 h in the dark. The precipitate that formed was filtered off, washed with a little *NN*-dimethylformamide and dried *in vacuo* [Found (mean of two determinations) C, 49.7; H, 5.2; N, 7.1; Zn, 16.8%]. The complexes formed from bilirubin and zinc [$\text{U-}^3\text{H}$]acetate or zinc [^{14}C]acetate were isolated in a similar fashion.

Discussion

When an excess of zinc acetate (or the zinc salts of other aliphatic carboxylic acids, e.g. propionate) in solution in dry *NN*-dimethylformamide or dimethylsulphoxide is added to a solution of bilirubin in the same solvent an intense red colour is formed immediately. Similar coloured solutions are formed with bilirubin and cadmium acetate or cobaltous acetate. Zinc acetate and mesobilirubin or bilirubin dimethyl ester also give coloured solutions under these conditions. The formation of the red complex is inhibited by the presence of acetic acid or water, no colour being formed if more than 20% (v/v) of water is present. The organic material recovered from samples of the *NN*-dimethylformamide–bilirubin (or bilirubin dimethyl ester)–zinc acetate complex that had been decomposed by water was identical with the starting tetrapyrrole in all respects.

By using the method of continuous variations, the molar ratio of zinc to bilirubin in these complexes was shown to be 3:1. Elemental analysis mentioned above for the *NN*-dimethylformamide–bilirubin–zinc acetate complex indicated a Zn:N ratio of 1:2, and samples of this complex that had been prepared from zinc [^{14}C]acetate (specific radioactivity 4.6×10^7 c.p.m./mmol) had radioactivities of 4.66×10^4 c.p.m./mg (mean of six determinations, corrected for quenching). Taken with the analysis results given above, this mean radioactivity corresponds to the presence of two acetate ions in a molecule whose

Table 1. Spectroscopic properties of rubin-metal acetate complexes in dipolar aprotic solvents

Tetrapyrrole	Solvent	Salt added	$\lambda_{\max.}$ (nm)	ϵ (l. mol ⁻¹ · cm ⁻¹)
Bilirubin	<i>NN</i> -Dimethylformamide	—	448	58700
	<i>NN</i> -Dimethylformamide	Zinc acetate	526	58000
	<i>NN</i> -Dimethylformamide	Cadmium acetate	536	—
	<i>NN</i> -Dimethylformamide	Cobaltous acetate	542	—
	Dimethyl sulphoxide	—	454	59200
	Dimethyl sulphoxide	Zinc acetate	528	63000
Mesobilirubin	<i>NN</i> -Dimethylformamide	—	420	50300
	<i>NN</i> -Dimethylformamide	Zinc acetate	482	64900
	Dimethyl sulphoxide	—	427	53200
	Dimethyl sulphoxide	Zinc acetate	485	55800
Bilirubin dimethyl ester	<i>NN</i> -Dimethylformamide	—	445	58700
	<i>NN</i> -Dimethylformamide	Zinc acetate	464, 505	—

molecular weight is 1041, and hence the complex has bilirubin : zinc : acetate : *NN*-dimethylformamide molar proportions of 1:3:2:2. Mass-spectrometric investigations of this orange solid at ambient temperature with direct insertion of the sample gave little information. When the temperature of the sample in the mass spectrometer was raised *NN*-dimethylformamide was evolved at 50°C and acetic acid at 100–120°C. The *NN*-dimethylformamide–bilirubin–zinc [U-²H]acetate complex behaved similarly, evolving C₂H²H₃O₂ at 100–120°C. Under similar conditions in the mass spectrometer zinc acetate dihydrate evolved acetic acid less readily than did the complex.

It was not possible to record the ¹H n.m.r. spectrum of the *NN*-dimethylformamide complex, since, once isolated, it is very insoluble. The spectrum of the [²H₆]dimethyl sulphoxide–bilirubin–zinc [U-²H]–acetate complex in [²H₆]dimethyl sulphoxide differed from the n.m.r. spectrum of bilirubin in the same solvent in that the signal at 6.09 p.p.m. due to the methine bridge protons had diminished in size.

Further evidence that the complex was a distinct chemical entity was provided by X-ray powder crystallography. Diffraction bands differing from those obtained with zinc acetate dihydrate or bilirubin alone were obtained, indicating that the complex was microcrystalline and was a definite compound.

It has not proved possible to isolate distinct complexes from solutions of bilirubin and zinc acetate in

other dipolar aprotic solvents. Cadmium acetate and cobaltous acetate give coloured solutions when added to bilirubin in *NN*-dimethylformamide. However, these complexes are less stable than their zinc analogues. No bathochromic shifts in the absorption spectra of solutions of bilirubin in *NN*-dimethylformamide or dimethyl sulphoxide could be detected when the acetates of the following metals were added: Li, Na, K, Be, Mg, Ca, Ag, Mn(II), Ni(II), Cu(II), Hg(I), Hg(II), Tl(I), Pb(II).

The microcrystalline and sparingly soluble nature of the *NN*-dimethylformamide–bilirubin–zinc complex has made determination of its structure difficult. One possible structure is shown in Table 1. These compounds provide further evidence that bilirubin can form complexes with Zn²⁺.

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