Direct Evidence for the Acid-catalysed Isomeric Scrambling of Bilirubin IX-α

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Summary Treatment of bilirubin IX-α in dimethyl sulphoxide with acetic acid at 85° or with concentrated hydrochloric acid at room temperature causes isomerisation and formation of bilirubin III-α and bilirubin XIII-α whereas similar treatment at room temperature with sodium hydroxide or sodium methoxide does not.

IT has been suggested,[1] on the basis of indirect evidence, that bilirubin IX-α (**1**) can undergo a reversible acid-catalysed cleavage about the central methylene bridge leading to isomeric scrambling and formation of bilirubin III-α (**2**) and bilirubin XIII-α (**3**). A similar isomerisation has also been postulated to occur under strongly basic conditions during the sodium amalgam reduction of bilirubin to mesobilirubinogen.[2,3] Our interest in the origin of the III-α and XIII-α isomers of bilirubin which are found, together with bilirubin IX-α, in some commercial samples of bilirubin[4] has led us to examine more closely the tendency of bilirubin IX-α to isomerise during acid or base treatment. We report here the first direct evidence for the acid-catalysed isomerisation of bilirubin IX-α and show that similar isomeric scrambling does *not* occur under strongly basic conditions.

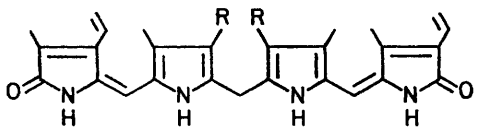
Solutions of bilirubin IX-α† in dimethyl sulphoxide (Me_2SO) containing glacial acetic acid, concentrated hydrochloric acid, aqueous sodium hydroxide, or methanolic sodium methoxide were kept in dim light under nitrogen for various time periods at 85° or at room temperature (22°) (Table). Dilution of the acidic solutions with water precipitated crude bilirubin which was collected, washed (H_2O, MeOH), and dried. Bilirubin was recovered from the basic solutions by extraction ($CHCl_3$) after prior neutralization with acetic acid; the chloroform extracts were washed (H_2O, 0·1M-$NaHCO_3$) and evaporated to give the product. Bilirubin isomers in the crude products were quantitatively separated by t.l.c.[4]‡ and their relative proportions were determined spectrophotometrically. This method of analysis is not ideal since, as previously reported,[4] a small reproducible amount of isomeric scrambling occurs as the pigments are applied to the thin layer plate, presumably due to catalysis by silica gel. Therefore only relative percentages of the III and XIII-α isomers in excess of 3 and 5% respectively should be considered to be significant.

Our results (Table) show that whereas there is no significant formation of the III and XIII-α isomers when bilirubin IX-α is heated in Me_2SO alone or kept at room temperature in Me_2SO–HOAc for 1 h, small amounts of both isomers are generated on heating bilirubin IX-α in Me_2SO–HOAc. Treatment of bilirubin IX-α in Me_2SO with concentrated HCl results in *immediate scrambling*, and the isomer composition of the product closely approaches the theoretical value of 25% III-α: 50% IX-α: 25% XIII-α expected for complete randomisation. When solutions in Me_2SO–HCl are allowed to stand there is a progressive decrease in the yield of recoverable bilirubin due to various side reactions. Correspondingly there is a marked change in the isomer composition of the product reflecting the differing stabilities of the three bilirubin isomers under these conditions. *exo*-Vinyl[5] substituents apparently have a destabilising influence.

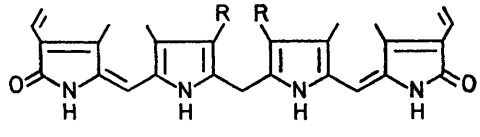
Effect of conditions on the isomerisation of bilirubin IX-α

Medium[a]	Time (min)	Reaction Temperature (°C)	Yield of bilirubin (%)	Relative % of bilirubin isomers in product III-α	IX-α	XIII-α
Me_2SO	110	85	80	2	95	3
Me_2SO–HOAc, 9:1 ..	60	22	96	3	93	4
Me_2SO–HOAc, 9:1 ..	60	85	96	9	81	10
Me_2SO–conc. HCl, 8:1 ..	1	22	87	23	49	29
Me_2SO–conc. HCl, 9:1 ..	*ca.* 2	22	74	16	48	36
Me_2SO–conc. HCl, 9:1 ..	15	22	76	8	39	54
Me_2SO–conc. HCl, 9:1 ..	30	22	62	6	33	61
Me_2SO–conc. HCl, 9:1 ..	60	22	42	<1	26	74
5M-NaOH in Me_2SO–H_2O, 1:1[b]	30	22	[c]	1	96	4
1M-NaOMe in Me_2SO–MeOH, 1:1	30	22	98	3	93	4

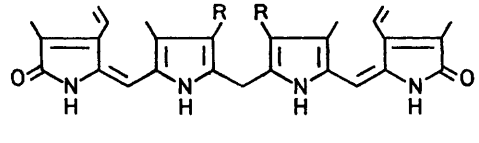
[a] Initial bilirubin concentration 5 mg/ml in all cases except for the control reaction in Me_2SO (row 1) in which the concentration was 2·5 mg/ml. [b] Biphasic mixture, stirred rapidly. [c] Not determined.



(1) Bilirubin IX-α



(2) Bilirubin III-α



(3) Bilirubin XIII-α

R = $-CH_2CH_2CO_2H$

Under alkaline conditions there was no evidence for isomerisation of bilirubin IX-α.

Common methods for the preparation of biliverdin IX-α invariably involve oxidation of bilirubin IX-α at *ca.* 95° under acidic conditions.[1,6–9] It is now clear that these methods are all bound to yield isomerically heterogeneous products. Similarly the use of MeOH–HCl to methylate bilirubin[8] will yield material which is isomerically impure.

Three isomeric verdins were obtained by Stoll and Gray[2] and by O'Carra and Colleran[3] on ferric chloride oxidation of mesobilirubinogen obtained by sodium amalgam reduction of bilirubin. It was presumed that isomerisation had occurred during the alkaline reduction step. Since bilirubin does not disproportionate under strongly alkaline conditions the observed isomerisation must have occurred during the acidic oxidation step, and it is therefore apparent

† The bilirubin IX-α used as starting material had ϵ_{max} 61,000—62,000 and contained less than 3 and 5% respectively of the III and XIII-α isomers.

‡ Isomers were identified on the basis of their isible absorption spectra and by t.l.c. comparison with authentic samples.[4]

that urobilinogens also undergo acid-catalysed isomerisation analogous to bilirubin, a property which is not shared by the closely-related urobilins.

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