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The Ready Isomerization of Bilirubin IX-α in Aqueous Solution

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The bile pigment bilirubin is obtained commercially from animal bile or gallstones. Since bile and gallstones are considered to contain derivatives of only one isomer of bilirubin, the IX-α isomer (Gray *et al.*, 1958), commercial preparations of bilirubin have also been assumed to be isomerically homogeneous (Petryka, 1966). It was recently found, however, that commercial bilirubin contains variable amounts of bilirubin III-α and bilirubin XIII-α in addition to the 'natural' IX-α isomer (McDonagh & Assisi, 1971). The origin of these two additional isomers is obscure, and at present it is not clear whether they are natural products or artifacts of the isolation procedures. Unlike bilirubin IX-α, they cannot be direct products of haem catabolism because their substituent sequence differs from that in protoporphyrin IX. A possible way by which they could arise, either *in vivo* or *in vitro*, is by cleavage of bilirubin IX-α at the central methylene bridge with subsequent recombination of the separate dipyrrole units to give mixtures of all three isomers. Bilirubin IX-α undergoes this type of isomeric scrambling under strongly acidic conditions (Bonnett & McDonagh, 1970; McDonagh & Assisi, 1972), but isomerization of bilirubin IX-α has not been demonstrated under less rigorous, 'physiological', conditions.

We have examined the isomeric stability of bilirubin IX-α under a variety of conditions representative of those that the pigment may encounter physiologically or during its isolation. In this communication we demonstrate that bilirubin IX-α disproportionates to form the III-α and XIII-α isomers in dilute aqueous solution within the approximate pH range 7.4–12. Free bilirubin IX-α readily isomerizes at physiological pH and temperature, but albumin-bound bilirubin IX-α does not.

Materials and methods

Materials. Organic solvents were analytical reagent grade. Tris (Trizma base) and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Phosphate buffer was prepared by the addition of 0.2M-NaH_2PO_4 to 0.2M-Na_2HPO_4, and 0.05M-tris buffer was prepared from 0.2M-tris base by adjusting the pH to 8.5 with 0.2M-HCl and diluting the solution fourfold.

Samples of bilirubin purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and from BDH Biochemicals, Poole, Dorset, U.K., were found to contain only trace amounts of bilirubins III-α and XIII-α. These samples, purified as follows, were used as the source of bilirubin IX-α. A mixture of bilirubin (400mg) and chloroform (450ml) was heated until the solvent boiled and then was cooled to room temperature. The solution (which contained some undissolved solid) was washed with 0.1M-$NaHCO_3$ solution (3×100ml), dried over anhydrous Na_2SO_4 (10g) and filtered. About one-third of the volume of chloroform was removed by distillation, and methanol was added in small portions to the boiling solution until a faint turbidity appeared. The solution was cooled to room temperature, and after 2h the crystalline precipitate was collected, washed with chloroform–methanol (1:1, v/v) and dried. The material thus obtained had $\epsilon_{max.}$ 61700–62300 in chloroform and the following isomeric composition: bilirubin III-α, 2%; bilirubin IX-α, 95%; bilirubin XIII-α, 3%. Bilirubin III-α and bilirubin XIII-α were obtained by t.l.c. of a mixture of the III-α, IX-α and XIII-α isomers (McDonagh & Assisi, 1971, 1972).

Bilirubin solutions were handled under subdued artificial light in a windowless room.

Isomerization of bilirubin IX-α. Bilirubin IX-α (5mg) was dissolved in 0.1M-NaOH (0.3–1.0ml) that had been purged with N_2 for 10min. This solution was immediately added from a narrow-tipped pipette to the rapidly stirred aqueous medium [100ml of 0.2M-phosphate buffer, 0.05M-tris buffer, water, 0.1M-NaOH, 5% (w/v) albumin in 0.2M-phosphate buffer or 5% (w/v) albumin in 0.05M-tris buffer], and the final solution was incubated for 2h at 37°C in an open flask immersed in a shaking water bath (100 oscillations/min). In one experiment bilirubin IX-α (5mg) was dissolved directly in 0.1M-NH_3 (100ml) and the solution was incubated as above. After incubation, the solutions were cooled to room temperature and bilirubin was isolated by one of two methods.

(1) Method 1, for protein-free solutions. The pH of the solution was adjusted to 4–5 with acetic acid to precipitate the pigment and stop the reaction. The mixture was extracted with chloroform (20, 20 and 10ml) and the extracts were washed (2×50ml of water, then 1×50ml of 0.1M-$NaHCO_3$), filtered,

combined and evaporated under reduced pressure to give the product.

(2) Method 2, for solutions containing albumin. Acetone (100–150ml) was added in portions to the stirred solution. The supernatant solution was decanted from precipitated protein, filtered through a pad of cottonwool and extracted once with 0.8–1.5 times its own volume of chloroform (before the extraction, 1ml portions of the solution were shaken with various ratios of chloroform to determine the minimum volume of chloroform required to give clear phase separation). The chloroform extract was washed with water (2×100ml) and 0.1M-$NaHCO_3$ (100ml), filtered and evaporated under reduced pressure to give the product.

For a control experiment, bilirubin IX-α (4.9mg) was dissolved in 0.3ml of N_2-purged 0.1M-NaOH. This solution was added to 0.1M-sodium acetate, pH4.5 (100ml), and the precipitated bilirubin was isolated by Method 1.

Isomerization of bilirubins III-α and XIII-α. Bilirubin III-α (0.47mg) and bilirubin XIII-α (0.48mg) were mixed with 0.06ml of N_2-purged 0.1M-NaOH, and a few drops of 0.05M-tris buffer, pH8.5, were added to give a clear solution. The solution was added to 0.05M-tris buffer, pH8.5 (20ml), and incubated aerobically for 2h at 37°C. The product was isolated by Method 1.

As a control, a mixture of bilirubin III-α (0.47mg) and bilirubin XIII-α (0.48mg) dissolved in chloroform (20ml) was shaken with 0.1M-$NaHCO_3$ (20ml). The aqueous phase was separated and the aqueous layer washed with chloroform (10ml). The phases were filtered, combined and evaporated, and the isomeric composition of the residue was analysed.

Product analysis. The recovery of bilirubin from each experiment was estimated spectrophotometrically in chloroform assuming $\epsilon_{max.} = 62600$, and the isomeric composition of each product was determined by using analytical t.l.c. and spectrophotometry as previously described (McDonagh & Assisi, 1971, 1972).

Results and discussion

Incubation of bilirubin IX-α aerobically at 37°C at low concentration (59μM) and at pH values 7.4–7.6 and 8.4–8.6 caused extensive isomerization and formation of the III-α and XIII-α isomers (Table 1). Despite rigorous attempts to keep the reaction conditions constant there was some variation both in the yield and isomeric composition of the product obtained at a given pH. At pH7.4–7.6 this probably was due partly to precipitation of bilirubin, which generally occurred during the incubation and occasionally within a few minutes of preparing the solution. Nevertheless recovery of bilirubin was generally high and significant amounts of the non-IX-α isomers always were formed at pH7.4–7.6 and 8.4–8.6. At these pH values the limiting isomer proportions appeared to be approximately 1:2:1 (III-α:IX-α:XIII-α). A control experiment (Table 1) indicated that isomerization did not occur during the preparation of the solutions or during the product isolation. Further, the observed isomer proportions were not due to selective precipitation of bilirubins III-α and XIII-α or to selective destruction of bilirubin IX-α in the starting material, since product recoveries were generally high.

Extensive isomerization was also observed at pH10–11, but above pH11 isomerization was diminished (Table 1). In 0.1M-NaOH there was extensive decomposition of the bilirubin and the product yield was low; in this case the isomer analysis may not reflect the degree of isomerization.

In marked contrast with the ready isomerization observed in neat aqueous buffer, bilirubin IX-α retained its isomeric identity when incubated under similar conditions in the presence of an excess of bovine serum albumin (Table 1). The reaction is therefore prevented by binding of the pigment to albumin.

Solutions of bilirubin IX-α in 0.1M-NaOH or 0.1M-Na_2CO_3 are frequently used during clinical analyses or biochemical studies. We therefore examined the stability of bilirubin in these media. When bilirubin IX-α (3mg/ml) was dissolved in 0.1M-NaOH, pH12.7, and kept for 30min at room temperature in the dark, significant isomerization was not observed. However, under similar conditions in 0.1M-Na_2CO_3, pH11.3, some isomerization did occur and the product (85% yield) was found to have the following isomeric composition: bilirubin III-α, 7%; bilirubin IX-α, 84%; bilirubin XIII-α, 10%.

As expected, the isomerization reaction is reversible. This was demonstrated by incubating a mixture containing bilirubin III-α and bilirubin XIII-α at pH8.5 (Table 2). After the incubation the recovered bilirubin was found to contain 44% of bilirubin IX-α. A control reaction showed the absence of bilirubin IX-α from the starting materials and in addition indicated the reliability of the analytical procedure used in this work.

When bilirubin is isolated from bile, bilirubin conjugates must first be hydrolysed before extraction of the free acid. If hydrolysis is done at pH7.4–10, as in some commercial processes (see, e.g., Porsche & Solms, 1945), it seems likely on the basis of the present findings that isomerization will occur and that the isolated product will be a mixture of isomers. However, hydrolysis at high pH should not lead to isomerization. In agreement with this, bilirubin isolated from human bile by the procedure of Ostrow *et al.* (1961), in which hydrolysis is effected with 0.1M-NaOH (20min, room temperature), was found

Table 1. *Isomerization of bilirubin IX-α to bilirubins III-α and XIII-α in aqueous media*

Bilirubin IX-α (5mg; isomeric purity 95%) was incubated for 2h at 37°C in the tabulated media. Bilirubin was recovered from each solution as described in the text and its isomeric composition determined by t.l.c.–spectrophotometry (McDonagh & Assisi, 1971). In the control, bilirubin IX-α (5mg) in 0.1M-NaOH (0.3ml) was added to 0.1M-sodium acetate (100ml); the precipitated bilirubin was extracted (chloroform) and analysed for isomers. The results in the two top lines are mean values (±S.D.) based on six and five separate experiments respectively.

Medium	pH	Recovery of bilirubin (%)	Relative proportions of bilirubin isomers in product (%)		
			III-α	IX-α	XIII-α
0.2M-Phosphate buffer	7.4–7.6	81±9	19±3	58±7	23±4
0.05M-Tris buffer	8.4–8.6	85±12	20±2	53±5	26±3
0.3mM-NaOH	9.9	79	22	52	25
1mM-NaOH	11.0	86	15	67	18
0.1M-NH_3	11.4	66	4	89	6
0.1M-NaOH	12.7	12	3	80	16
5% (w/v) serum albumin in 0.2M-phosphate buffer	7.3	30	4	90	6
5% (w/v) serum albumin in 0.2M-phosphate buffer	7.6	36	3	93	5
5% (w/v) serum albumin in 0.05M-tris buffer	8.5	79	2	94	4
Control		93	2	94	5

Table 2. *Isomerization of bilirubins III-α and XIII-α to bilirubin IX-α*

A mixture of bilirubin III-α (0.4mg) and bilirubin XIII-α (0.48mg) was incubated in 0.05M-tris buffer, pH8.5 (20ml), for 2h at 37°C. Bilirubin was isolated from the solution as described in the text and its isomer composition determined by t.l.c.–spectrophotometry (McDonagh & Assisi, 1971). For a control, the isomer composition of a similar mixture in chloroform was analysed in the same way.

	Recovery of bilirubin (%)	Relative proportions of bilirubin isomers (%)		
		III-α	IX-α	XIII-α
Composition of initial mixture		49	0	51
After incubation	66	20	44	36
Control in chloroform	—	44	2	54

to contain less than 2% each of the III-α and XIII-α isomers. Similar results were found for bilirubin isolated from rat bile by the same procedure.

The data suggest that the relatively large amounts of bilirubin III-α and XIII-α found in some commercial samples of bilirubin are artifacts. This conclusion does not exclude, however, the possibility that small amounts of the non-IX-α isomers occur naturally in bile, gallstones and other natural materials. Indeed, the results do indicate that isomerization of bilirubin IX-α might occur *in vivo*, although not in serum, where the pigment is bound to albumin. Unfortunately, the analytical procedure we have used cannot be employed to demonstrate the complete absence of isomers from samples because a small amount of isomerization invariably occurs during the t.l.c. separation (McDonagh & Assisi, 1971, 1972). It may be noted here that in the rat all three isomers are rapidly excreted into the bile at comparable rates after intravenous injection (A. F. McDonagh, F. Assisi & G. Kirshenbaum, unpublished work).

The mechanism of the isomerization reaction is not yet clear. However, it is evident that at some stage the bilirubin molecule must fragment into dipyrroles, which can then recombine to give bilirubin isomers or possibly react to give dipyrrole products. A similar cleavage mechanism may be

involved in the conversion of bilirubin IX-α into dipyrrolic structures *in vivo*.

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