COMMERCIAL BILIRUBIN: A TRINITY OF ISOMERS

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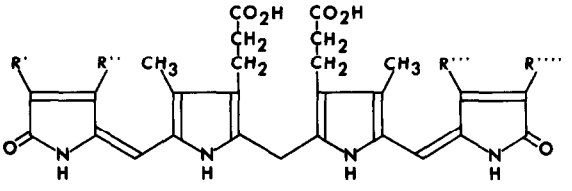
1. Introduction

Commercial preparations of the bile pigment, bilirubin, are used (frequently without further purification) in metabolic investigations, as a convenient starting material for the preparation of other bile pigments, as standards for clinical analyses, and in studies of the chemistry of bilirubin itself. The commercial material, which is obtained from bile or gallstones of bovine or porcine origin, is generally believed to consist essentially of bilirubin IX-α (fig. 1B) formed metabolically via stereospecific cleavage of the α-methene bridge of protohaem-IX and the only isomer of bilirubin known to occur naturally. Although evidence has been published [1] suggesting that commercial bilirubin contains trace amounts of other isomers (bilirubin IX-β and -δ) arising from cleavage of photohaem at the β- and δ-methene bridge positions, more recent work [2] has shown these additional isomers comprise less than 0.5% of bilirubin isolated from bile. The latter observation, coupled with results from earlier studies using oxidative degradation techniques [3], forms the basis for the notion that bilirubin derived from natural sources has an isomeric purity (IX-α) of > 99%.

We have found that bilirubin from a number of commercial sources is separable by thin layer chromatography (TLC) into three components, and present here unequivocal evidence that these are bilirubin III-α (A), bilirubin IX-α (B), and bilirubin XIII-α (C), isomeric bilirubins [4] with the structures shown in fig. 1.



	R'	R''	R'''	R''''
A	$CH=CH_2$	CH_3	CH_3	$CH=CH_2$
B	CH_3	$CH=CH_2$	CH_3	$CH=CH_2$
C	CH_3	$CH=CH_2$	$CH=CH_2$	CH_3

Fig. 1. Structures of bilirubin III-α (A), IX-α (B) and XIII-α (C).

2. Materials and methods

TLC plates (20 X 20 cm) were prepared using silica gel H (E. Merck). Plates were activated (60 min at 120°), cooled to room temperature in air, and stored over silica gel dessicant in a closed chamber; plates were generally used within 2–24 hr after activation. Bilirubin was applied as a filtered solution in $CHCl_3$ (1 mg/ml). Plates were developed (1–2 hr) in closed tanks containing 100 ml developing solvent and lined internally with filter paper saturated with solvent. For analytical work layers were 0.25 mm thick (solvent, 1% glacial CH_3COOH in AR-$CHCl_3$). For preparative work layers 0.5 mm thick were used; plates were activated, washed with methanol overnight by continous development, dried, and reactivated as above. Bilirubin solution (up to 400 μl/plate) was applied as a streak using a Cordis Microapplicator and the plates

were developed with 2% glacial CH_3COOH in AR-$CHCl_3$. Separated products were eluted from the adsorbent with $CHCl_3$. Eluates were washed (H_2O, 0.1 M $NaHCO_3$), filtered and evaporated to dryness in flasks coated internally with "Siliclad" to prevent loss of bilirubin by adsorption to glass [5].

3. Results

Of the several samples of bilirubin examined, that supplied by Pfanstiehl Laboratories, Inc., (ϵ_{max} = 61,700 in $CHCl_3$) contained the least bilirubin IX-α (< 65%). On the other hand, this material provided the richest source of the III- and XIII-α isomers and was used as starting material for their isolation.

On TLC Pfanstiehl bilirubin showed three major yellow components with close R_f values (fig. 2), in addition to minor amounts of other more polar impurities which were not examined. Preparative TLC of this material (73 plates; on 16 of these resolution of the two lower components was unsatisfactory and only the top band was collected) followed by crystallization of the products from $CHCl_3$-MeOH, gave bilirubin III-α (A, top band, 6 mg; $\lambda_{max}^{CHCl_3}$ 455–458 nm, ϵ_{max} 65,200), bilirubin IX-α (B, middle band, 18 mg; $\lambda_{max}^{CHCl_3}$ 453–455 nm, ϵ_{max} 62,600), and bilirubin XII-α (C, lower band, 4 mg; $\lambda_{max}^{CHCl_3}$ 449–453 nm, ϵ_{max} 52,500). The products decomposed on heating without melting (234–275°) and were shown to be essentially homogenous by TLC (fig. 2). Three-fold crysallization ($CHCl_3$-MeOH) of the starting material caused no evident improvement in its homogeneity.

The products were shown to be isomers of molecular formula $C_{33}H_{36}N_4O_6$ by high resolution mass spectrometry [A, B, and C, M^+ (found) = 584.262 ± 0.002; M^+ (calc.) = 584.263]. Their low resolution mass spectra were alike, and similar to spectra previously recorded for bilirubin IX-α [6, 7]. In particular, each spectrum showed an intense pair of dipyrrylmethene fragment ions at m/e 286 and 299 arising from cleavage about the central methylene bridge.

Chromic acid oxidation [8] of A, B, and C yielded methylvinylmaleimide in yields of 29, 34, and 44% respectively but methylethylmaleimide was not detected, demonstrating the absence of ethyl side-chains and the presence in each compound of rings bearing both methyl and vinyl substituents.

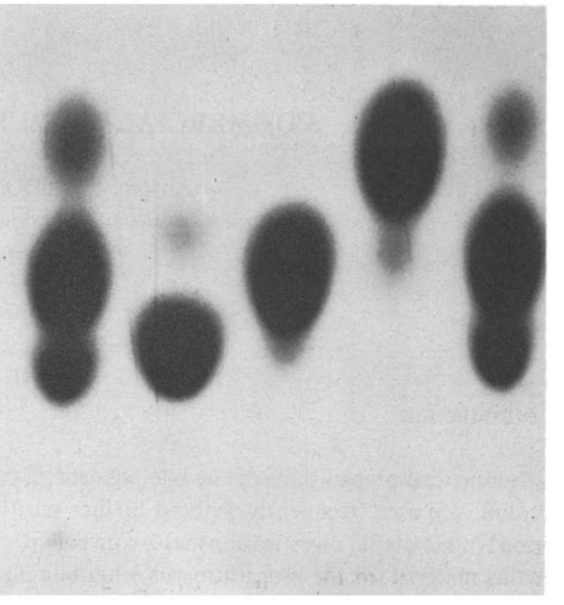


Fig. 2. Thin layer chromatography of bilirubin. L. to r. Pfanstiehl bilirubin, purified bilirubin XIII-α (C), purified bilirubin IX-α (B), purified bilirubin III-α (A), 3× crystallized Pfanstiehl bilirubin.

The absorption spectra of A, B, and C are qualitatively similar, but the bathochromic and hyperchromic trend from C to B to A was indicative of increasing conjugation in the same sequence.

These observations suggested that A, B, and C are bilirubin III-, IX, and XIII-α, respectively. This conclusion was proved by their conversion to the corresponding biliverdin dimethyl esters by oxidation with benzoquinone in DMSO-CH_3COOH followed by methylation [9]. Biliverdin III-α dimethyl ester was obtained from A, biliverdin IX-α dimethyl ester from B, and biliverdin XIII-α dimethyl ester from C in respective yields of 36, 44, and 43% (identification of the products was based on visible absorption spectra, melting points and TLC data). Oxidation of a concentrated solution of B in DMSO-CH_3COOH (4.4 mg/ml) yielded a mixture of bilirubin III-, IX-, and XIII-α due to acid-catalyzed isomerization of the starting material [9].

Because of its unsymmetrical substitution sequence, bilirubin IX-α yields two isomeric azo-pigments on treatment with diazotized ethyl anthranilate and these are separable by TLC (silica gel; EtOAc-$CHCl_3$, 1:1)

[10]. In contrast, the symmetrically substituted III- and XIII-α isomers should each give a unique azo-pigment. Diazotization of B with ethyl anthranilate gave two azo-pigments; A gave one azo-pigment, having the same R_f as the *more polar* of the pair obtained from B; and C also gave one azo-pigment, having the same R_f as the *least polar* of the pair from B. This result, besides confirming the structures of A, B, and C, enables structures to be assigned absolutely to the isomeric azo-pigments isolated by Compernolle et al. [10].

Under the conditions used for TLC, bilirubin IX-α disproportionates to give trace amounts of the III-α and XIII-α isomers. This disproportionation is a complicating factor which must be recognized if TLC is used for analyses of bilirubin isomer mixtures. Preparative TLC experiments using purified bilirubin IX-α showed that the artifactual mixture of isomers thus formed contained 1% III-α: 95% IX-α: 4% XIII-α.

Qualitative examination of bilirubin samples from 15 different vendors by analytical TLC and quantitative analysis of 5 of these by preparative TLC indicated the presence of non-IX-α isomers in most of these samples in amounts ranging from trace quantities to as much as 16% III-α: 62% IX-α: 22% XIII-α for material from Pfanstiehl Laboratories.

4. Discussion

The discovery that commercial bilirubin may contain isomers other than IX-α, and in particular isomers which could not be directly derived from protohaem-IX, raises the question of their origin. Either they are artifacts of the commercial extraction processes and arise from acid or base-catalyzed isomerization of bilirubin IX-α, or they were naturally present in the original starting material. Both of these possibilities are currently under investigation. It should be noted that III-, IX-, and XIII-α bile pigment isomers are not distinguishable by the oxidative degradation techniques [3, 11] which have been used as monitors of isomeric homogeneity in bile pigment research.

Puzzling aspects of bilirubin chemistry have been the discordant range of extinction coefficients which have been recorded for the "pure" pigment [12] and the consequent difficulty of obtaining the compound sufficiently pure for a clinical analytical standard [13, 14]. The work reported here provides at least a partial explanation for the former, and suggests a method for accomplishing the latter. Other implications of this work will be discussed elsewhere.

Acknowledgements

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